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Ghrelin: A Growth Hormone-Releasing Factor in Birds.

By



Sameera Ahmed

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science.

Department of Physiology

Edmonton, Alberta

Fall 2001

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Ghrelin: A Growth Hormone-Releasing Factor in Birds** submitted by **Sameera Ahmed** in partial fulfillment for the degree of Master of Science.

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DEDICATION



To my family....

...“The Ahmed’s”.

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ABSTRACT

Ghrelin, a recently discovered peptide in the mammalian hypothalamus and gastrointestinal tract is thought to be the endogenous ligand for the growth hormone (GH)-secretagogue (GHS) receptor, and it stimulates GH release in rats and humans. The possibility that ghrelin is present in birds was therefore assessed, since a GHS receptor is present in the chicken pituitary gland.

Although immunoreactive ghrelin is readily detectable in rat stomach and ileum, ghrelin-immunoreactivity could not be detected in the chicken gastrointestinal tract. Ghrelin immunoreactivity was, however, present in the chicken hypothalamus, although not in the arcuate (infundibular) nucleus as in rats. Discrete magnocellular cells and neuronal fibres with ghrelin immunoreactivity were present in the chicken hypothalamus. In preliminary experiments, ghrelin markedly increased GH release in immature chickens; with a GH response comparable to that induced by human GH-releasing hormone (GHRH), although less than that induced by thyrotropin-releasing hormone (TRH). Ghrelin also stimulated GH release from dispersed pituitary cells *in vitro*, indicating that its GH-releasing action *in vivo* is at least partially mediated by direct pituitary action.

Early embryonic growth is thought to be a 'growth without GH' syndrome, since it occurs prior to the ontogenetic differentiation of the pituitary gland and thus in the absence of pituitary GH. However, immunoreactive GH is almost ubiquitously present in extrapituitary tissues of early chick embryos, and extrapituitary GH may be a paracrine/autocrine regulator of early embryogenesis. The regulation of extrapituitary GH

expression during embryogenesis is, however, unknown. The possibility that extrapituitary GH may be dependent upon the local presence of ghrelin was, therefore, examined. Ghrelin-like proteins were also found within the neural tissues of chick embryos at embryonic day 7, of the 21-day incubation period. The distribution of ghrelin immunoreactivity was comparable to that of GH. Ghrelin may, therefore, function in an autocrine/paracrine manner to regulate the release of extrapituitary GH during early embryogenesis.

These results demonstrate the presence of a ghrelin-like protein in the hypothalamus of neonatal chicks and in neural and non-neural tissues of ED 7 chick embryos and suggest the participation of ghrelin in GH regulation in birds.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophin
AGRP	agouti-related peptide
AM	anterior hypothalamus nucleus
ARC	arcuate nucleus
BBB	blood brain barrier
Ca	caudal lobe (pituitary gland)
Ce	cephalic lobe (pituitary gland)
cp	choroid plexus
CNS	central nervous system
CO	optic chiasma nucleus
CSF	cerebrospinal fluid
d	diencephalon
DAB	diaminobenzidine
di	diocoel
ec	ectodermal layer
ED	embryonic day
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRP	growth hormone-releasing peptide
GHRP-R	growth hormone-releasing peptide receptor
GHS	growth hormone secretagogue

GHS-R	growth hormone secretagogue receptor
GPCR	G protein-couple receptor
Gr cells	ghrelin cells
GRF	growth hormone-releasing factor
hGHRH	human growth hormone-releasing hormone
ICC	immunocytochemistry
i.c.v.	intracerebroventricular
if	infundibulum
IGF-1	insulin growth factor-1
i.v.	intravenous
m	mesencephalon
ME	median eminence
mRNA	messenger ribonucleic acid
ms	mesodermal layer
MTLRP	motilin-related peptide
NPY	neuropeptide Y
ov	otic vesicle
PACAP	pituitary cyclase-activating peptide
PBS	phosphate buffered saline
Pit-1	pituitary specific transcription factor-1
PRL	prolactin
PRM	nucleus magnocellularis preopticus pars medialis

PRS	nucleus magnocellularis preopticus pars supraopticus
PVN	periventricular nucleus
RIA	radioimmunoassay
RNA	ribonucleic acid
RP	optic recess
SRIF	somatostatin
T ₃	tri-iodo-thyronine, thyroid hormone
tg	trigeminal ganglion
TRH	thyrotropin-releasing hormone
VIII	third ventricle

CHAPTER ONE

Literature Review



I. PREAMBLE

Following the conceptualization of neurosecretion and neuroendocrine function (Harris, 1948), the hypothalamus was thought to be primarily responsible for the regulation of the anterior pituitary gland. According to this hypothesis, each adenohypophyseal hormone was thought to be specifically regulated by a single factor [for instance, adenocorticotropin (ACTH) was specifically regulated by corticotropin-releasing factor (CRF); thyroid stimulating hormone (TSH) was specifically regulated by thyrotropin-releasing hormone (TRH)]. This hypothesis was later revised with the realization that a single releasing factor could regulate more than one pituitary hormone (for instance, luteinizing hormone-releasing hormone (LHRH) stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH); TRH stimulates the release of TSH and prolactin). The concept of *dual hypothalamic regulation* was further developed with the realization that hypothalamic neurosecretions could inhibit pituitary function (for instance, dopamine inhibits prolactin release). Dual hypothalamic regulation has traditionally provided a conceptual model for the control of growth hormone (GH) secretion; GH release from pituitary somatotrophs being increased by GH-releasing hormone (GHRH) and suppressed by somatostatin (somatotropin-release-inhibiting hormone, SRIF). This model is, however, outdated since a third hypothalamic factor, an endogenous ligand for a novel GH-secretagogue (GHS) receptor in the anterior pituitary, ghrelin, participates in the hypothalamic control of GH secretion. This model may, however, only be appropriate for mammalian species, since LHRH and TRH are more important growth hormone-releasing factors (GRFs) in fish and birds, respectively, than GHRH. The possible participation of ghrelin in GH secretion in a bird, the domestic fowl (*Gallus domesticus*), is the focus of this investigation.

II. GENERAL INTRODUCTION

It is now well established that growth hormone (GH) release from the anterior pituitary gland is stimulated by a hypothalamic growth hormone-releasing hormone (GHRH). A hypothalamic GH-releasing factor (GRF) was first postulated in the early 1960s, since lesions of the preoptic nuclei reduced pituitary GH concentrations in rats (Reichlin, 1961). However, it wasn't until 1982 that GHRH was isolated, sequenced and characterized; originally from pancreatic tumours in patients with acromegaly (Guillemin et al., 1982; Esch et al., 1982; Rivier et al., 1982), and subsequently from the hypothalamus of humans and other mammals (Bohlen et al., 1983; Brazeau et al., 1984; Vaughan et al., 1992; Parker et al., 1996).

A physiological role for GHRH in GH release has been demonstrated by the blockade of GH secretion following GHRH immunoneutralization (Wehrenberg et al., 1982; Frohman & Jansson, 1986; Giustina & Veldhuis, 1998; Sassolas, 2000). It is particularly important in triggering pulsatile or episodic GH release, although this also results from phasic inhibition of GH secretion by synchronous removal of somatostatin (SRIF) tone.

The interplay between GHRH and SRIF has classically been considered to provide the primary regulation of somatotroph function. However, a number of other hypothalamic factors (neuropeptides and neurotransmitters) also influence GH release by either stimulating GHRH at hypothalamic sites or by directly stimulating the pituitary gland (Muller, 1987) – (Tables 1.1 & 1.2). Amongst these are opioid peptides, which primarily stimulate GHRH release from the hypothalamus (Baile et al., 1986; Schanbacher, 1986). Synthetic analogs of these peptides led to the discovery of a hitherto unknown group of peptides, called GH-secretagogues (GHSs), that stimulate GH release via specific receptors (GHS-Rs) that utilize signal transduction pathways distinct from those activated by GHRH. The endogenous ligand for this receptor has now been identified and this peptide, 'ghrelin' (*ghre* = "to grow"), is thought to participate in the tripartite regulation of GH release in mammals (Kojima et al., 1999; Bowers, 2001).

Table 1.1: GH-releasing neuropeptides in mammals.

GH-releasing neuropeptide	Site of action		Interspecies variations	
	Pituitary	CNS	Rat	Human
<u>GHRH-like peptides</u>				
Vasoactive intestinal peptide	*	*	+	+
Secretin		*	+	?
Glucagon		*		+
Pituitary adenylate cyclase activating polypeptide.	*	*	+	+
Gastric inhibitory peptide		*	+	?
<u>GHRH-unrelated peptides</u>				
Thyrotropin-releasing hormone	*		+	+
Luteinizing hormone-releasing hormone	*		+	+
Angiotensin II	*	*	+	
Bombesin		*	+ in females	?
Gastrin		*	ineffective	+ in males
Cholecystokinin		*	+	ineffective
Neurotensin		*	+	?
Substance P	*	*	+/-	+
Motilin	*		+	?
Galanin	*	*	+	+
Neuropeptide Y	*	*	+/-	?
Vasopressin		*	ineffective	+
Adrenocorticotropin & Melanocyte-stimulating hormone	*	*	+	+
Endogenous opioid peptides	*	*	+	+
Activin A	*	*	-	+
Phe-Met-Arg-Phe-amide		*	+	?
Delta Sleep-inducing peptide	*	*	+	?

* denotes the site of action of the neuropeptide

+ stimulatory effect on GH release

- inhibitory effect on GH release

? poorly understood effects

(Adapted from Harvey, 1995).

Table 1.2: Modulators of GH regulation in mammals.

GH-modulator	Interspecies variations	
	Rat	Human
<u><i>GH-regulating neurotransmitters</i></u>		
Acetylcholine (cholinergic muscarinic pathway)	+	+
Dopamine (dopaminergic pathway)	+	+
Catecholamines (alpha 2-adrenergic pathway)	+	+
Serotonin (serotonergic pathway)	+/-	+/-
Histamine (histamine pathway)	-	+
GABA (gamma-aminobutyric acid)	+	+
Excitatory amino acids (e.g. glutamate)	+	?
Nitric oxide	+/-	no effect
<u><i>GH-regulating metabolic substrates</i></u>		
Glucose	no change	-
Leptin	+	+
Amino acid (L-arginine)	no change	+
<u><i>Other GH-regulating hormones</i></u>		
Glucocorticoids	+/-	+/-
Testosterone	+	+
Estrogen	+	+
Thyroid hormones	+	+

+ stimulatory effect on GH release

- inhibitory effect on GH release

? poorly understood effects

(Adapted from Giustina & Veldhuis, 1998).

In non-mammalian species, GHRH and SRIF are also thought to regulate GH secretion (Harvey, 1993), although other hypothalamic factors may be of greater importance. In birds, for instance, thyrotropin-releasing hormone (TRH) is a physiological GRF, with greater GH-releasing activity than mammalian GHRH peptides (Harvey, 1983; 1990). Moreover, although a GHRH gene is expressed in the chicken hypothalamus (McRory et al., 1997), its role in GH secretion is still uncertain (Harvey, 1999; 2000). The possible presence or participation of a ghrelin-like peptide in the regulation of avian GH secretion is currently unknown and the aim of the studies proposed.

III. GROWTH HORMONE-RELEASING HORMONE: A GROWTH HORMONE-RELEASING FACTOR IN BIRDS?

A. GHRH Structure

Growth hormone-releasing hormone (GHRH) peptides have been cloned and sequenced from the hypothalamus of humans (Guillemin et al., 1982; Rivier et al., 1982; Ling et al., 1984), pigs (Bohlen et al., 1983), cows (Esch et al., 1982), goat and sheep (Brazeau et al., 1984), hamsters (Ono et al., 1994), rats and mice (Spiess et al., 1983; Frohman et al., 1989) and fish (Vaughan et al., 1992; Parker et al., 1996). In all cases, the mature peptide (a linear peptide) is composed of approximately 40 amino acids. In mammals, the biological activity of these peptides reside within a shorter fragment GHRH₍₁₋₂₉₎ of the full molecule, since the removal of additional amino acids from the N- or C-terminal end of the fragment, significantly reduces the GH-releasing activity of the peptide in mammals (Frohman & Jansson, 1986; Smith et al., 1999b).

In birds, the presence of a hypothalamic GHRH was first indicated by the destruction of the arcuate nucleus (principal site of GHRH production in mammals) by monosodium glutamate or electrical lesioning, which reduced plasma GH in chickens (Scanes & Cammarotto, 1982; Rabii et al., 1984). Moreover, extracts of chicken, turkey, pigeon and duck hypothalami induced GH release when co-incubated with pituitary

tissue (Harvey et al., 1979a,b; Harvey, 1983). The avian pituitary gland would thus appear to be dependent upon a hypothalamic GRF that stimulates GH release in birds, as in mammals.

A novel chicken GHRH gene was subsequently cloned and sequenced (McRory et al., 1997). The chicken GHRH (cGHRH) sequence was reported to code for a 46 amino acid peptide possessing 42% sequence identity with human GHRH but 74% sequence identity with fish GHRH. This peptide did not, however, induce GH secretion when systemically injected into anesthetized chickens (Harvey, 1999). This may, however, reflect a sequencing error, since the sequence initially reported had an asparagine at position 21 [i.e. cGHRH(Asp)²¹] whereas lysine is conserved in all other GHRH peptides, and is thought to be required for biological activity (Wehrenberg & Ling, 1983; Baile et al., 1986; DeAlmeida & Mayo, 1998). This possibility is supported by later studies (Sherwood et al., 2000) that confirmed the sequence originally reported was erroneous and demonstrated that cGHRH(Lys)²¹ had biological activity; increasing cAMP production in chick neuroblasts (Erhardt et al., 2001) and chicken pituitary glands (Toogood et al., 1999). The GH-releasing activity of this peptide is, however, still unknown.

B. GHRH gene family

In all species, the GHRH peptide belongs to a superfamily of neuropeptides with structural homology [other members include, secretin, vasoactive intestinal peptide (VIP) and pituitary cyclase-activating peptide (PACAP)] (Monts et al., 1996; Gaylinn, 1999). However, the structural organization of the GHRH gene differs between mammals and sub-mammalian vertebrates (Montero et al., 2000). In chickens, for instance, the GHRH gene also encodes for PACAP (*grf/pacap*) (McRory et al., 1997), whereas, PACAP is encoded on a separate gene (*pacap*) in mammals (Monetro et al., 2000). A single gene encoding GHRH and PACAP is also found in fish (McRory et al., 1997; Parker et al., 1996; Fradinger & Sherwood, 2000; Sherwood et al., 2000) and in amphibia (Alexandre et al., 2000). This indicates gene duplication during the molecular evolution of the GHRH/PACAP gene family (Montero et al., 2000). Interestingly, cPACAP is 97%

identical to hPACAP, thus while GHRH sequences are poorly conserved throughout evolution, PACAP sequences are strongly conserved during phylogeny (Arimura, 1998).

C. GHRH gene expression

In mammals, GHRH is expressed predominantly in the arcuate nucleus and in the periventricular nucleus of the hypothalamus (Frohman et al., 1989; Katakami & Matsukura, 1992; Petersenn et al., 1998). The presence of the GHRH gene in the hypothalamus of chickens was initially indicated using a cRNA probe for rat GHRH (Harvey et al., 1991). The recent cloning and sequencing of the cGHRH gene in the chicken brain (McRory et al., 1997; Sherwood et al., 2000) reveals the gene undergoes alternative splicing, producing a 46 amino acid peptide, cGHRH₁₋₄₆, (most abundant form), a 43 amino acid form, cGHRH₁₋₄₃, (due to exon sliding), and a cGHRH₃₃₋₄₆, form (due to exon skipping), which is biologically inactive.

In addition to the hypothalamus, the GHRH gene is also present in the pituitary, pancreas, kidney, lung, testis, ovary, adrenal gland, heart and extra-hypothalamic structures in mammals (Brar et al., 1989; Suhr et al., 1989; Moretti et al., 1990; Srivastava et al., 1993), and in neuroblast cells of ED 3.5 chick embryos, as well as in the ovary and testis of chickens (McRory et al., 1997; Erhardt et al., 2001).

D. GHRH distribution

1. Hypothalamus

GHRH-immunoreactive perikarya have been detected within the hypothalamus of mice (Miki et al., 1996), rats (Bloch et al., 1983a; Jacobowitz et al., 1983; Merchenthaler et al., 1984; Sawchenko et al., 1985; Bruhn et al., 1987; Lantos et al., 1995), bats (Mikami et al., 1988; Anthony et al., 1991), monkeys (Bloch et al., 1983; Lechan et al., 1984) and humans (Bloch et al., 1983; Frohman & Jansson, 1986). The presence of GHRH proteins in avian species has yet to be demonstrated, since studies with antisera against mammalian GHRH failed to immunocytochemically label hypothalamic nuclei in chickens (Harvey et al., 1991).

In all mammalian species, GHRH- perikarya are primarily localized in the arcuate nucleus (infundibular nucleus in humans). Neurons within the arcuate nucleus send axonal projections to the median eminence, where GHRH-immunoreactive fibres terminate on hypophysial portal vessels (Frohman & Jansson, 1986; Anthony et al., 1991; Bertherat et al., 1992; Leshin et al., 1994; Takahashi et al., 1995; Rao et al., 1996; Romero & Phelps, 1997; Miller et al., 1999). Some GHRH-immunostained cell bodies are also localized in the paraventricular nucleus, suprachiasmatic nuclei, and ventromedial nucleus in rats (Leidy & Robbins, 1988; Fodor et al., 1994). Other GHRH-immunoreactive fibres, which terminate in the periventricular nucleus and ventral dorsomedial, are seen to reciprocally innervate the arcuate nucleus in bats and rodents (Sawchenko et al., 1985; Leidy & Robbins, 1988; Anthony et al., 1991; Fodor et al., 1994)

2. Extrahypothalamic regions

Apart from GHRH neurons located within hypothalamic structures of the mammalian brain, GHRH-containing perikarya are also present in the cortical and subcortical telencephalon, and in the cerebral cortex, amygdala, hippocampus and cerebellum (Leidy & Robbins, 1988; Anthony et al., 1991; Takahashi et al., 1995). It is speculated that GHRH has roles unrelated to GH regulation in these brain regions, such as in sleep, memory, learning and feeding (Campbell & Scanes, 1992).

3. Peripheral tissues

GHRH has also been detected in tissues other than the brain. For instance, GHRH immunoreactivity has been found in gonadal tissue. It is present in thecal and granulosa cells of the ovary and in the leydig and sertoli cells of the testis (Berry & Pescovitz, 1988; Moretti et al., 1990; Ciampani et al., 1992; Bagnato et al., 1992; Breyer et al., 1996). Other sites of GHRH distribution include the pancreas, the gastrointestinal tract, the immune system and the placenta, although the roles of GHRH in these tissues are uncertain (Bosman et al., 1984; Weigent et al., 1991; Petersenn et al., 1998; Gaylinn, 1999). GHRH is also produced in various neoplasms, including breast, endometrial and

ovarian cancers, in which locally produced GHRH may directly affect tumour cell proliferation (Losa et al., 1990; Kahan et al., 1999).

E. GHRH action

1. Somatotroph proliferation and differentiation

GHRH contributes to the cell-specific differentiation of GH-secretory cells of the anterior pituitary gland and increases their size (Harvey, 1995). A reduction in somatotroph number is, therefore, observed in GHRH deficient states or GHRH resistance (Harvey, 1995). GHRH stimulates the phenotypic differentiation of GH-secreting cells and it induces 'silent' somatotrophs to become GH-secreting cells during ontogeny (Porter et al., 1995; Piper & Porter, 1997). The somatotrophic action of GHRH is antagonized by SRIF, since somatotroph size, nuclear volume and GH content is increased if the hypothalamic release of SRIF is blocked (Harvey, 1995).

2. GH gene expression and synthesis

In mammals, GHRH is a major physiological stimulus for GH secretion and synthesis, since GH gene transcription is increased by GHRH (Harvey, 1995). GH gene expression in the anterior pituitary gland is also increased by the stimulatory effects of GHRH in birds (Radecki et al., 1994). GHRH increases GH mRNA accumulation in the cytoplasm, and accumulation of the GH protein in somatotrophs *in vitro* and *in vivo* (Harvey, 1995). GH gene transcription is stimulated within 10 min of exposure to GHRH *in vivo* but requires 2-3 days of continuous GHRH stimulation *in vitro* (Harvey, 1995). In birds, the synthesis of GH mainly occurs in somatotrophs of the caudal lobe and GH gene expression is induced by GHRH in caudal lobe somatotrophs, but not in those in the cephalic lobe (Kansuku et al., 1995).

3. GH release

It is well established that the destruction of the hypothalamus in mammals and birds, suppresses plasma GH concentrations (Scanes & Cammarotto, 1982; Rabii et al., 1984; Root, 1989; Miller et al., 1999). Conversely, stimulation with exogenous GHRH

rapidly increases GH release in all vertebrates (for reviews see Frohman & Jansson, 1986; Giustina & Veldhuis, 1998). In addition to direct effects on pituitary somatotrophs, GHRH may regulate GH secretion through a complex interplay with SRIF (Richardson & Twente, 1992; Tannenbaum, 1994; Giustina & Veldhuis, 1998). GHRH and SRIF are reciprocally regulated in mammals (Aguila & McCann, 1985; Murakami et al., 1987; Hindmarsh et al., 1991; West et al., 1997a,b). Consequently, i.c.v. administration of GHRH decreases GH secretion in rats, whereas the i.c.v administration of SRIF increases peripheral plasma GH concentrations (Murakami et al., 1987; Tannenbaum, 1994). SRIF neurons synapse on GHRH neurons in the arcuate nucleus (Liposits et al., 1988; Horvath et al., 1989; McCarthy et al., 1992; Tannenbaum et al., 1998a), and SRIF promotes GHRH release from hypothalamic tissue *in vitro* (West et al., 1997; Giustina & Veldhuis, 1998).

Continuous or repetitive GHRH exposure *in vivo* initially increases plasma GH concentrations, but is followed by diminished responsiveness of the pituitary somatotroph in mammals and in birds (Buonomo et al., 1987; Harvey et al., 1985; Scanes & Harvey, 1985); a phenomenon termed homologous desensitization (Bilezikian & Vale, 1984; Aleppo et al., 1997). A similar phenomenon occurs *in vitro* and is independent of GH depletion (Vance et al., 1985; Reichardt et al., 1996; Miller et al., 1999; Thorner, 1999). Intermittent exposure of somatotrophs to GHRH over several days, conversely, improves pituitary responsiveness to GHRH compared to continuous infusion; a phenomenon known as potentiation (e.g in mammals - Petersenn et al., 1998; Gaylinn, 1999 and in birds - Scanes & Harvey, 1985).

The potent GH-releasing ability of GHRH clearly demonstrates it is a major regulator of GH secretion in mammals. Mammalian GHRH peptides also increase plasma GH concentrations in avian species (Leung & Taylor, 1983; Scanes et al., 1984, 1986; Radecki et al., 1994), but a higher dose-level is required to elicit GH responses than in mammals (Harvey et al., 1991). The maximum dose of mammalian GHRH that elicits a GH response in birds is between 1-10ug/kg, with intermediate doses being more effective than higher doses (Harvey & Scanes, 1984). GHRH-induced GH release in birds is also temporally biphasic, since the release of GH is followed by a period of refractoriness

(Harvey et al., 1985; Scanes & Harvey, 1984). The maximal GH response occurs 5-10 minutes after GHRH is intravenously injected into birds. This rapid GH response suggests GHRH directly stimulates avian somatotrophs, probably by the activation of pituitary GHRH receptors (Toogood et al., 1999).

In birds, hGHRH₁₋₄₀ is far more potent in eliciting a GH response than hGHRH₁₋₂₉ (Scanes et al., 1986). C-terminal residues may therefore be important in receptor-binding and stimulation of GH release in birds (Leung & Taylor, 1983; Scanes et al., 1986).

The possibility that GHRH is physiologically involved in GH release in birds is supported by its antagonism by SRIF (Taylor et al., 1986; Scanes & Harvey, 1989), insulin growth factor -1 (IGF-1) and thyroid hormones, which have been shown to be physiological inhibitors of GH release (Buonomo et al., 1987; Harvey et al., 1991). However, while mammalian GHRH peptides have GH-releasing activity in birds, the GH releasing activity of chicken GHRH is uncertain (Harvey, 1999).

IV. THYROTROPIN-RELEASING HORMONE: A GROWTH HORMONE-RELEASING FACTOR IN BIRDS?

A. TRH structure

Thyrotropin-releasing hormone (TRH) was the first hypophysiotropic peptide to be isolated and chemically synthesized (Boler et al., 1969). The structure of the tripeptide is pGlu-His-ProNH₂, and was termed TRH due to its stimulatory effects on thyrotropin (TSH) release from the pituitary gland in all vertebrates (Nelson, 1982). However, in addition to regulating TSH release, TRH is a potent GRF in mammals, birds, reptiles, amphibia and fish (Harvey, 1990).

B. TRH gene

TRH gene expression, in birds, predominantly occurs in the hypothalamus and the pituitary gland (Guissouma et al., 1998). Transcription of hypothalamic and pituitary

TRH is regulated by thyroid hormone (Tri-iodo-thyronine, T₃) (Guissouma et al., 1998, 2000). It has been demonstrated that T₃ decreases transcription from the TRH promoter, whilst hypothyroidism significantly increases TRH transcription in chickens (Guissouma et al., 1998, 2000).

A number of TRH gene-related peptides are also encoded by the TRH gene. In rats, processing of the TRH precursor results in the generation of not only TRH, but also a number of 'cryptic' peptides (Bulant et al., 1988). These cryptic peptides flank five TRH progenitor sequences, and two of them (termed P₄ and P₅) modulate the *in vivo* GH response in chickens to TRH (Harvey & Cogburn, 1996; Geris et al., 2000c).

C. TRH distribution

1. Hypothalamus

TRH is primarily produced in the paraventricular nucleus (PVN) in the avian hypothalamus (Griffiths et al., 1982; Peczely & Kiss, 1988; Jozsa et al., 1988; Geris et al., 1999). Immunoreactive TRH is also localized in the median eminence (ME), with smaller amounts of TRH immunoreactivity being present in the periventricular hypothalamic nuclei (PHN), preoptic nuclei (POP), anterior medialis nuclei (AM), ventromedial nuclei (VMN) and the dorsomedial hypothalamic nuclei (DMN) of the chick brain (Geris et al., 1999). TRH is, therefore, likely to be transported from these hypothalamic nuclei to the portal vessels leading to the pituitary gland. TRH-immunoreactivity is also detected in the avian pituitary gland for autocrine/paracrine actions on GH release (Geris et al., 1998b, 1999).

2. Extrahypothalamic regions

Immunoreactive TRH is also present in extrahypothalamic regions of the avian brain. A significant concentration of TRH, measured by radioimmunoassay, is present in the brain stem, cerebellum, optic lobes and telencephalon of the chick brain (Geris et al., 1999).

3. Peripheral tissues

TRH is also present in various peripheral tissues, although at concentrations much lower than in the brain (Geris et al., 1999). TRH is, for instance, present in the thyroid gland and gonads (ovary and testis) of chickens, for the regulation of thyroidal and reproductive function (Geris et al., 2000b). Smaller amounts of TRH immunoreactivity have also been detected in the gastrointestinal tract (stomach, duodenum, rectum), bursa of fabricius, kidney, heart, lung, pancreas, liver, spleen, muscle and skin (Geris et al., 2000b). This indicates TRH has possible roles in digestive, immune and respiratory processes, unrelated to its neuroendocrine roles in GH regulation.

D. TRH action

1. Somatotroph proliferation and differentiation

TRH not only promotes GH synthesis, but also increases the number of pituitary cells secreting GH *in vitro* (Dean et al., 1997). The increase in the number of GH-secreting cells *in vitro* can be detected 2h after stimulation, and is thought to induce the possible recruitment of 'silent' or 'non-responding' somatotrophs (Hull & Harvey, 2000). Similar to GHRH, TRH causes a reduction in 'apparent' somatotroph number and GH content within 10-30 min after stimulation *in vivo* (Hull & Harvey, 2000). This occurs due to the rapid degranulation of GH-secretory granules and depletion of detectable GH, and is not due to cell death (Hull & Harvey, 2000).

2. GH gene expression & synthesis

TRH, similar to GHRH, also promotes GH synthesis in birds *in vitro* and *in vivo* (Kansaku et al., 1995; Hull & Harvey, 2000). The increase in GH synthesis and release by TRH indirectly impairs subsequent somatotrophic activity, since peripheral actions of GH cause an increase in circulating T₃ and IGF-1 levels, and T₃ exerts a direct inhibitory effect on the accumulation of newly synthesized GH in chickens (Denver & Harvey, 1991). This is in contrast to the effects of T₃ in rats, since thyroid hormone has a stimulatory action on GH synthesis (Samuels et al., 1989). Interestingly, SRIF only

blocks TRH-induced GH release and does not affect TRH-induced GH synthesis or storage (Barinaga et al., 1985).

3. GH release

It is well established that TRH is a physiologically important GRF in birds (Klandorf et al., 1985; Harvey & Baidwan, 1989; Harvey, 1990, 1993; Harvey et al., 1991, 1997). A physiological role for TRH is suggested by the antagonism of TRH-induced GH release by SRIF (Harvey, 1990; Harvey et al., 1997). Somatotropic function is also reduced after passive TRH immunoneutralization (Klandorf et al., 1985). TRH rapidly increases GH secretion in chickens (Harvey et al., 1990; Scanes et al., 1981; Proudman, 1984), turkeys (Proudman & Opel, 1981), geese (Scanes et al., 1979) and ducks (Foltzer-Jourdainne et al., 1988). TRH stimulates GH release *in vivo* and *in vitro* with more potency than mammalian GHRHs (Leung & Taylor, 1983; Harvey et al., 1990; Scanes & Harvey, 1988). Dose levels of TRH required to elicit a GH response in birds are lower than those needed to stimulate thyroid function, and are comparable with those found in the hypophysial portal circulation (Harvey & Scanes, 1984; Harvey, 1990). TRH has also been shown to possess stimulatory effects in both late embryonic development and early post-hatch development (Perez et al., 1987; Harvey, 1990, 1993). This mirrors an ontogenetic rise in the TRH content of the chicken hypothalamus (Geris et al., 1998b).

In chickens, the GH response to TRH is rapid and is followed by a period of refractoriness to further stimulation *in vivo* (Scanes & Harvey, 1988). This can, however, be overcome by exposure to another provocative stimuli (Harvey et al., 1985). Conversely, chronic incubation of chicken pituitary glands with TRH continuously provokes GH release *in vitro* (Harvey et al., 1985; Dean et al., 1997). The difference in GH response to stimulation with TRH *in vitro* and *in vivo* can be explained by GH refractoriness, which involves the interactions of TRH and SRIF at hypothalamic sites (Harvey et al., 1991). TRH stimulates SRIF release at central sites, and SRIF blocks TRH-induced GH release as with GHRH-induced GH release in birds (Scanes & Harvey, 1989; Harvey, 1993). TRH may thus modulate somatotroph responsiveness to TRH

stimulation *in vivo*, especially since TRH-induced GH release provides a short-loop negative feedback mechanism at hypothalamic sites (Lea et al., 1990).

The GH-releasing activity of TRH is not only modulated by TRH itself but also by TRH gene-related peptides (Cheng et al., 1986; Harvey et al., 1997). Both P₄ and P₅ suppress basal and TRH-induced GH secretion *in vivo* in chickens, but have no effect on pituitary glands *in vitro*, suggesting a central site of action (Harvey et al., 1997). In contrast, P₄ potentiates the actions of TRH in rats (Bulant et al., 1988). A tripeptide (pGlu-Glu-ProNH₂), with structural similarities to TRH, also interacts with TRH and inhibits TRH-induced GH release *in vitro* but potentiates the GH response *in vivo* in chickens (Harvey, 1993). TRH metabolites are also known to influence the GH response to TRH. The metabolism of TRH results in the production of two metabolites 1) deamido-TRH (TRH-OH) and 2) diketopiperazine (DKP), which also possess GH-releasing activity (Cheng et al., 1986). Both TRH-OH and DKP interact synergistically with TRH at hypothalamic sites, and TRH-OH stimulates basal and TRH-induced GH release *in vitro* but DKP antagonizes TRH *in vitro*. Thus, these metabolites differentially modulate TRH action at hypothalamic and pituitary levels (Decuypere & Scanes, 1983; Harvey et al., 1997).

TRH and GHRH have a synergistic relationship, since co-administration of these peptides *in vivo* induces a synergistic GH response in birds. However, TRH is far less potent in stimulating GH release *in vitro*; TRH-induced GH release is thus partly mediated by actions at central sites (Harvey & Scanes, 1984; Taylor et al., 1986), possibly by the stimulation of GHRH from the hypothalamus (Harvey & Scanes, 1985; Taylor et al., 1986; Buonomo et al., 1987; Foltzer-Jourdainne et al., 1988; Harvey et al., 1990)

A. Introduction

1. *Growth hormone-releasing peptides (GHRPs)*

In the 1970s, it was discovered that structural modifications of metenkephalin led to the production of synthetic peptides capable of GH release *in vitro* and *in vivo* (Bowers et al., 1991; Ghigo et al., 1997; Bowers, 1998; Camanni et al., 1998; Ghigo et al., 1999). Molecules such as arginine and morphine were known to elicit GH secretion; however, they were incapable of direct pituitary somatotroph stimulation and functioned by altering endogenous GHRH secretion (Monts et al., 1996; Camanni et al., 1998). Conversely, opioid-derived peptides were found to directly stimulate somatotrophs with great potency (Camanni et al., 1998), and these compounds were termed growth hormone-releasing peptides (GHRPs). Artificial GHRPs were synthesized before the isolation of GHRH (Guillemin et al., 1982) and were thought to mimic a hypothalamic GRF, although the subsequent isolation of GHRH revealed no structural homology with GHRPs (Camanni et al., 1998). These potent GHRPs were thus thought to mimic another natural GRF involved in GH regulation (Elias et al., 1995; Bowers, 1996; Ghigo et al., 1997; Smith, 1998; Bowers, 1998).

2. *Peptidomimetics*

Aside from peptide GHRPs, non-peptidyl GHRPs or peptidomimetics were also developed, and found to be even more potent than the artificial peptides in releasing GH in mammals (Ghigo et al., 1997; Casanueva & Dieguez, 1999). The existence of biologically active GHRPs and peptidomimetics, collectively termed growth hormone secretagogues (GHSs), indicate that GH regulation is complex and may involve several GRFs (Bowers, 1998; Smith et al., 1998; Kojima et al., 1999).

B. Structure

1. *Growth hormone-releasing peptides (GHRPs)*

The basic structure of the first family of synthetic GHRPs was: Tyr-d-Tyr-Gly-Phe-Met-NH₂ (DTrp2) (Bowers, 1993; Monts et al., 1996; Ghigo et al., 1997; Smith et al., 1998). Three other chemical types of GHRPs, consisting of 4 or 5 amino-acids, were immediately synthesized and included: Tyr-Ala-D-Trp-Phe-Met-NH₂ (DTrp3), Tyr-D-Trp-D-Trp-Phe-NH₂ (DTrp2,3) and Tyr-D-Trp-Ala-Trp-D-Phe-NH₂ (DTrp2, LTrp4). However, these synthetic peptides exhibited GH-releasing abilities *in vitro* but could not elicit pituitary GH release *in vivo* (Elias et al., 1995; Deghenghi, 1998). Nevertheless, these structures became templates for further GHRPs that have proven to be more potent than the initial family of GHRPs. This second generation of GHRPs includes the hexapeptide (GHRP-6) (His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂), and two different hexapeptides, GHRP-2 (DAla- D β Nal-Ala-Trp-D-Phe-Lys-NH₂) and Hexarelin (His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂), and a heptapeptide (GHRP-1)(Ala-His-D β Nal-Ala-Trp-D-Phe-Lys-NH₂) (Elias et al., 1995; Massoud et al., 1996; Bowers, 1999).

2. *Peptidomimetics*

Peptidomimetics are non-peptidyl GHRP synthetic compounds that have been extensively studied as therapeutic agents for clinical GH dysfunctions, such as in dwarfism and familial short stature (Laron et al., 1995; Klinger et al., 1996; Schally & Comaru-Schally, 1998; Casanueva & Dieguez, 1999). These GHRP mimetics possess the advantage of being orally bioavailable in greater amounts (>60%), compared to GHRP analogs (<1%), due to their non-peptide components that are not immediately degraded by peptidases (Walker et al., 1990; Ghigo et al., 1997; Deghenghi, 1999; Smith et al., 1999b; Hashizume et al., 1999; Hansen et al., 1999). Various peptidomimetics have been synthesized and include L-692, 429, L-692, 585, L-700, 653, L-163, 191 (or MK-0677) and mimic the actions of an endogenous GRF (Elias et al., 1995; Ghigo et al., 1997; Raun et al., 1998).

C. GHRP/GHS Receptor

1. *Introduction*

Initial attempts to characterize the GHRP-receptor (GHRP-R) were challenging due to the low number of binding sites and low GHRP-R mRNA abundance in the pituitary (Howard et al., 1996; Smith et al., 1996). Recently, however, the GHRP-R/GHS-R has been cloned and characterized in humans (Howard et al., 1996), pigs (Pong et al., 1996; Van der Ploeg et al., 1998), rats (Yokote et al., 1998) and chickens (Toogood et al., 1999; Gaylinn et al., 2000). In general, GHSs bind to GHS-Rs with high affinity, although GHRPs and peptidomimetics bind with different affinities (Van der Ploeg et al., 1998; Chen et al., 1998; Howard et al., 1999). Furthermore, the binding of GHRP-6 is competitively inhibited by other GHRP analogs but not by GHRH (Pong et al., 1996; Smith et al., 1999a). This indicates that GHRH and GHSs function via distinct receptors (Blake & Smith, 1991).

2. *GHS-R: A new family of GPCRs*

The GHS-R is a member of a newly discovered family of G-protein coupled receptors that are not related to any other known G-protein coupled receptors (Smith et al., 1999a,b). The GHS-R contains 7 putative alpha-helical membrane-spanning segments with 3 intracellular and 3 extracellular loops (Howard et al., 1999). Moreover, a highly conserved motif responsible for G protein interaction is present in the second intercellular loop and cystenes found in the first two extracellular loops are capable of disulphide bonding (Howard et al., 1999). The GHS-R identified by Howard et al., (1996) is a 41kDa receptor, although a 57kD GHS-R was found in human, bovine and porcine pituitary glands (Ong et al., 1998), providing evidence for the presence of receptor subtypes. Three subfamilies (FMs) of the GHS-R have also been identified (Muccioli et al., 1997, 1998). FM1 receptors are expressed in the pituitary gland, thyroid gland, stomach and bone marrow, FM2 receptors are expressed in the brain, and FM3 receptors are expressed in the heart, spleen, lung, liver, skeletal, muscle, kidney and gonads (Smith et al., 1999b).

3. GHS-R gene expression & distribution

Molecular analysis of the human GHS-R revealed that it had been strongly conserved in evolution (Howard et al., 1999). A single, highly conserved, gene encodes the GHS-R with a protein sequence that is 96% identical in humans and rodents (Yokote et al., 1998; Smith, 1998; Smith et al., 1999b). Structurally distinct GHRPs activate both the puffer fish (*Fugu rubripes*) and human GHRP-R, thus the structure and function of the GHRP-R ligand-binding pocket and the natural endogenous ligand has been conserved for 400 million years (Smith et al., 1999a; Palyha et al., 2000). The presence of highly homologous GHRP-R/GHS-Rs in birds and in mammals, suggests the existence of an unknown naturally occurring ligand that is an important component of the GH regulatory system in these species (Monts et al., 1996; Camanni et al., 1998; Smith et al., 1999b), especially as the GHS-R is expressed in the pituitary of both vertebrate groups. The receptor is also known to be expressed in the arcuate- and ventromedial-hypothalamic nuclei, and in other brain regions and body organs of mammals (Table 1.3). The binding of GHRPs to GHS-Rs also occurs with kinetics that is comparable across all species studied (Dickson et al., 1995; Ghigo et al., 1997; Bennett et al., 1997; Yokote et al., 1998; Smith et al., 1999a,b; Shuto et al., 2001).

D. GH-releasing actions of GHSs

1. Actions of GHSs *in vitro*

GHRH and GHSs act differently on different somatotroph sub-populations. GHSs increase the number of GH-secreting cells without altering GH content in each cell, whereas GHRH increases both the number and amount of GH being released from the pituitary cells (Badger et al., 1984; Wu et al., 1994; Monts et al., 1996; Raun et al., 1998; Smith et al., 1999a). The GH-releasing abilities of GHSs are lower *in vitro* compared to GHRH (Badger et al., 1984; Ghigo et al., 1997; Ghigo et al., 1998). Different mechanisms of action mediating GHS and GHRH release are also indicated by their additive effects on GH release (Ghigo et al., 1997; Bowers, 1998; Ghigo et al., 1998). GHRP-6 and GHRH act in an additive manner in monkeys, cows and in man (Bowers et al., 1990; Massoud et al., 1996a; Bowers, 1998).

Table 1.3: Tissue distribution of the growth hormone secretagogue (GHS)-receptor (GHS-R).

Tissue	Species	Reference
Stomach	human	Date et al., 2000b
	rat	Kojima et al., 1999, Shuto et al., 2001
Pituitary	human	Ong et al., 1998
	cow	Ong et al., 1998
	pig	Howard et al., 1996,
	rat	Shuto et al., 2001
	mouse	Hosoda, 2000
	chicken	Gaylinn et al., 2000
Hypothalamus	human	Kojima et al., 1999
	rat	Kojima et al., 1999, Shuto et al., 2001
	human	Ghigo et al., 1997
	rat	Muccioli et al., 1997
	rat	Muccioli et al., 1997
	rat	Muccioli et al., 1997
Higher CNS regions	rat	Smith, 1998, Smith et al., 1999a
	rat	Smith, 1998, Smith et al., 1999a
	human	Ghigo et al., 1997
	human	Ghigo et al., 1997
	human	Ghigo et al., 1997
	human	Ghigo et al., 1997
	human	Ghigo et al., 1997
	human	Ghigo et al., 1997
Brain stem	rat	Muccioli et al., 1997
Heart	rat	Muccioli et al., 1997
Lung	rat	Muccioli et al., 1997
Kidney	rat	Mori et al., 2000
Liver	rat	Muccioli et al., 1997
Spleen	rat	Muccioli et al., 1997
Gonads	rat	Muccioli et al., 1997
Skeletal muscle	rat	Muccioli et al., 1997
Bone marrow	rat	Muccioli et al., 1997
Tumours	Adenomas (GH, PRL & ACTH secreting)	Korbonits et al., 2001a
	Somatotropinomas	Korbonits et al., 2001a
	Bronchial tumours	Korbonits et al., 2001a

2. Actions of GHSs *in vivo*

The GH-releasing activity of GHSs in rats is far greater in the presence of hypothalamic tissue than in its absence, hence GHSs are better GH-releasers *in vivo* than *in vitro* (Badger et al., 1984; Wu et al., 1994; Hickey et al., 1996; Ghigo et al., 1997; Chen et al., 1998; Smith et al., 1996b, 1998; Korbonits et al., 1999; Smith et al., 1999a). This fact is consistent with the widespread expression of the GHS-R gene in the CNS (Monts et al., 1996; Camanni et al., 1998; Smith et al., 1999a), and with the greater activity of GHS when administered centrally rather than peripherally in rats (Yagi et al., 1996), in guinea pigs and sheep (Smith, 1998), in goats (Hashizume et al., 1999) and in swine and dogs (Okada et al., 1996; Hansen et al., 1999).

Synergism between GHSs and GHRH is observed *in vivo*, since an intact hypothalamic-pituitary stalk is crucial for optimal GHS-induced GH secretion (Figure 1.1) (Mallo et al., 1992; Popovic et al., 1995; Jaffe et al., 1996; Bowers, 1998; Korbonits et al., 1995, 1998; Maheshwari et al., 1999). The synergism between the GHSs and GHRH is more marked *in vivo* than *in vitro*, since maximal and even sub-maximal effective doses of GHSs and GHRH *in vivo* have more than additive effects on GH release (Sartor et al., 1985; Cheng et al., 1989; Cheng et al., 1993; Wu et al., 1994; Oliver et al., 1999).

GHRH is required for maximal GH responses to GHSs (Pandya et al., 1998), because GHRH up-regulates GHS-R mRNA levels up to 200% in rats *in vivo* (Kineman et al., 1999). Furthermore, passive immunization against GHRH markedly blunts GHS action (Clark et al., 1989; Pandya et al., 1998). Thus, GHSs require GHRH to optimally release pituitary GH, which is proposed to occur through interactions of GHS-R on GHRH-containing neurons in the rat hypothalamus (Tannenbaum et al., 1998b; Bluet-Pajot et al., 2001). As GHRH acts centrally to inhibit its own secretion, GHSs are thus more potent than GHRH in stimulating GH release *in vivo*.

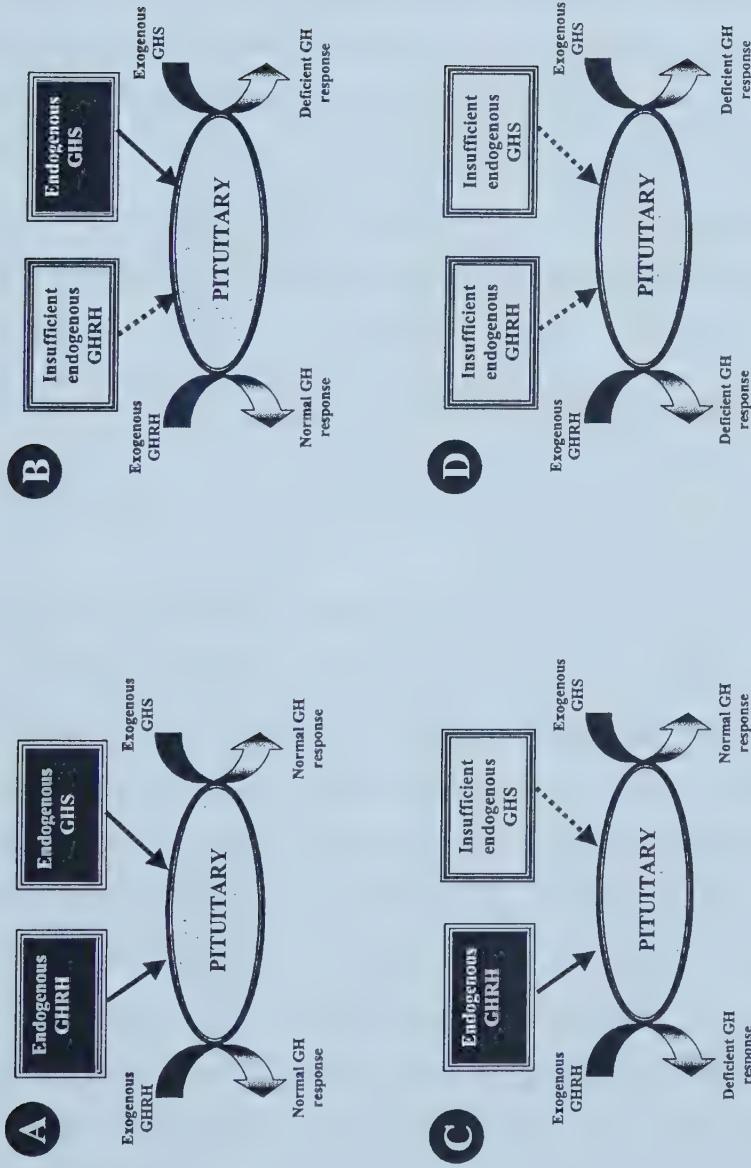


Figure 1.1: Schematic of the synergism between growth hormone-releasing hormone (GHRH) and the natural growth hormone secretagogue-receptor (GHS-R) ligand in GH regulation. (A) This hypothetical model in support of the complementary relationship of GHRH and the endogenous GHS-R ligand assumes that a 'normal' response to GHRH or GHS requires the presence of its endogenous analog (i.e. GHS-R ligand or GHRH respectively). (B & C) A blunted response to either exogenous GHRH or GHS is interpreted as indicating a deficiency of its endogenous complement. (D) Blunted responses to both exogenous GHRH or GHS administered implies deficiencies of both endogenous complements. (Adapted from Bercu & Walker, 1999).

2.1 Actions of GHS on GHRH neurons

Electrophysiological studies using rat (Dickson et al., 1993; Bailey & Leng, 1998) and monkey (Smith et al., 1999a) hypothalamic slices, have shown that GHSs activate neurons in the arcuate nucleus (ARC) and in the ventromedial- and periventricular nuclei (Dickson et al., 1997; Dickson, 1999; Bailey et al., 1998; Tannenbaum et al., 1998b; Hewson et al., 1999). It has therefore been hypothesized that the GHS-R ligand might act directly as a GHRH regulator (Guillaume et al., 1994; Fletcher et al., 1996; Dickson, 1999; Tannenbaum & Bowers, 2001), since anatomical evidence indicates these secretagogues directly modulate GHRH release (Smith et al., 1999a; Ghigo et al., 1999). The abundance of GHS-Rs on GHRH-containing neurons supports this possibility (Guan et al., 1997; Bennett et al., 1997). Furthermore, i.v administration of GHSs in sheep increase GHRH secretion into the hypophysial portal blood (HPB) by 2.5 fold for 45-60 min and in turn stimulate 2.3-5.3 fold increases in GH plasma levels (Guillaume et al., 1994; Fletcher et al., 1996; Oliver et al., 1999).

2.2 Actions of GHS on SRIF neurons

It was postulated that GHSs might influence GH secretion by modulating SRIF tone, via GHS-Rs on SRIF neurons in the PVN, although GHSs do not stimulate SRIF release into the HPB in sheep (Guillaume et al., 1994). GHSs may, however, locally suppress SRIF tone in GHRH-containing neurons in the arcuate nucleus (Goth et al., 1992; Dickson et al., 1993; Bellone et al., 1995; Conley et al., 1995; Massoud et al., 1997; Bailey & Leng, 1998; Ghigo et al., 1998; Smith et al., 1998; Smith et al., 1999b; Hewson et al., 1999). Infusion of GHSs in rats not only increase GH release but also disrupt rhythmical GH bursts, which are triggered by asynchronous changes in GHRH and SRIF release (Clark et al., 1988; Fairhall et al., 1995).

2.3 Actions of GHS on another putative hypothalamic hormone

The hypothalamic action of GHSs are complex and not fully understood. Therefore, it has been postulated that GHSs mediate their actions with the aid of a putative hypothalamic factor (u-factor), that is co-released with GHRH to maximize the GH response to GHSs (Bowers et al., 1984; Malozowski et al., 1991; Nargund & Van der Ploeg, 1997; Bowers, 1999). Indeed, it is thought that GHRH is the initiator of episodic GH release, whereas the hormone mimicked by GHSs is responsible for modulating the amplitude and duration of the GH pulse (Jaffe et al., 1993a,b; Fairhall et al., 1995; Oliver et al., 1999; Bowers, 1999).

2.4 Desensitization

GHSs, like GHRH, induce homologous desensitization both *in vitro* and *in vivo* (Jaffe et al., 1993a,b; Huhn et al., 1993; Ghigo et al., 1997; Ghigo et al., 1998) when continuously infused in rats (Maheshwari et al., 1999), sheep (Wu et al., 1994), pigs (Chang et al., 1995) and humans (Ghigo et al., 1998; Shah et al., 1999). This effect is less pronounced when GHSs are intermittently administered, and the desensitization is reversed by withdrawal of the treatment (Rahim et al., 1998). A negative feedback mechanism may also be involved, since the administration of GH blunts GHSs actions in humans (Arvat et al., 1997b).

2.5 Age-related actions

The actions of GHS on GH release are markedly age-related (Ghigo et al., 1997; Ghigo et al., 1998). For instance, GHSs stimulated GH secretion in the human fetal pituitary (18- and 31-week gestation), indicating the presence of a functional GHS-R in the fetus (Shimon et al., 1998). Both GHS and GHRH are able to induce GH release in newborn mammals, however, the GH response to GHRH is far greater than that induced by GHSs at this age (Bartolotta et al., 1997). Thereafter, the GH response to GHSs and GHRH is similar in pre-pubertal stages, at puberty and in young adulthood. A marked decline in the GH response to both GHRH and GHSs are seen with increasing age (Aloi

et al., 1994; Bellone et al., 1995, 1998; Arvat et al., 1999; Bowers & Granda-Ayala, 2001).

2.6 Gender-related actions

The enhanced GH-releasing activity of GHS found at puberty can be explained by interactions between the secretagogues and gondal steroids (estrogen and testosterone) (Bercu et al., 1991; Bellone et al., 1995; Loche et al., 1997). However, while estrogen and testosterone may amplify the GH response to GHSs, there is no significant decrease in somatotroph responsiveness in menopausal women (Bowers, 1993; Penalva et al., 1993; Arvat et al., 1999). The differing modulatory effects of gondal steroids on GHS-induced GH release observed at pre-pubertal, pubertal and early adult period may, alternatively, simply reflect age-related differences in responsiveness (Arvat et al., 1999).

2.7 Signal transduction pathway

Like GHRH and other GH secretagogues, GHSs signal through the protein kinase C pathway. Following GHRP binding to the receptor, phospholipase C and inositol triphosphate (IP₃) second messenger systems are activated with an accumulation of diacylglycerol and a re-distribution of calcium ions (Ca²⁺) within the somatotrophs, leading to GH release (Cheng et al., 1991; Akman et al., 1993; Herrington & Hille, 1994; Bresson & Dufy-Barbe, 1996; Monts et al., 1996; Ghigo et al., 1997; Smith et al., 1998; Smith et al., 1999a,b; Xu et al., 2000). GHSs also block potassium (K⁺) currents facilitating an influx of Ca²⁺ through voltage-gated L-type channels (Smith, 1998; Nass & Thorner, 1999). However, GHRP-2 functions differently to GHRP-6 and acts on voltage-gated Ca²⁺ channels to increase both transmembrane L- and T-type Ca²⁺ currents (Chen et al., 1998b, 1996). Hence, differing GHSs act through differing pathways, which could reflect the different GHS-R subtypes present in different species (Wu et al., 1996; Smith, 1998; Korbonits et al., 1998).

3. GH-releasing actions of GHSs in birds

The actions of L-692, 429 (a peptidomimetic) were examined in a study with chickens that revealed dual sites of GHS action (geris et al., 2001). There is a direct action on the pituitary, as well as a central site of action, which increases hypothalamic TRH release to promote GH secretion in the chicken (Geris et al., 1998a). This is consistent with TRH being an important and potent GH-releaser in avian species (Geris et al., 1998a; Harvey, 1999; Geris et al., 2000a,b) and is the first demonstration of GHSs stimulating TRH in any species. However, unlike its relationship with GHRH in mammals, no additive or synergistic relationship was seen between L-692, 429 and TRH. This probably reflects the fact that both secretagogues activate the PKC pathway in chickens. However, an additive increase in GH secretion was observed with L-692, 429 and GHRH when co-administered, as these GRFs mediate their effects through distinct pathways (Geris et al., 1998a).

Similar results were also obtained in the chicken with the GHS, L-163, 255, as it directly stimulated GH release at the pituitary level and had a central mode of action through an increase in TRH levels (Geris et al., 2001). Although these GHSs directly stimulate GH from the pituitary, it is thought that their main action occurs centrally. Neither L-692, 429 or L-163, 255 showed any effects on SRIF release in the birds, but SRIF pre-treatment markedly reduced somatotroph responsiveness to the GHSs.

4. PRL, ACTH & cortisol secretion

GHSs are not specific GH regulators; they also induce dose-dependent prolactin (PRL), adrenocorticotropin (ACTH) and cortisol secretion (Jacks et al., 1994; Arvat et al., 1997a; Ghigo et al., 1997; DeKeyzer et al., 1997; Loche et al., 1997; Ghigo et al., 1998; Bailey & Leng, 1998; Korbonits et al., 1998; Frieboes et al., 1999; Ghigo et al., 1999). GHSs stimulate lactotrophs to release PRL, and this response is not dependent upon age or gender (Bowers, 1993; Ghigo et al., 1997). GHSs also have a stimulatory effect on ACTH and cortisol levels in mammals. The maximal stimulatory dose required of GHS to induce ACTH and cortisol is much lower than that needed for the maximal GH response (Massoud et al., 1996b). The ACTH response to GHSs is independent of gender

but is related to age (Arvat et al., 1997a). GHSs also act centrally to stimulate ACTH and cortisol release, since no stimulation occurs in *in vitro* pituitary cell cultures nor after following hypothalamo-pituitary disconnection *in vivo* (Arvat et al., 1999).

5. Other actions of GHSs

Other unrelated roles for the GHS-R ligand have been speculated, since the GHS-R is also expressed in many regions of the rat brain that are not involved in GH regulation (Yokote et al., 1998; Ghigo et al., 1999; Smith et al., 1999a). GHS-Rs have been located in the rat hippocampus, dentate gyrus, anteroventral preoptic nucleus, substantia nigra, anterior hypothalamic area, and paraventricular nucleus (Smith, 1998; Smith et al., 1999a). GHS binding sites are also present in the human choroid plexus and cerebral cortex, suggesting GHSs participate in non-endocrine events (Ghigo et al., 1997).

5.1 Feeding behaviour

It has been demonstrated that GHRH increases food intake in rats when administered centrally (Vaccarino et al., 1985, 1988). Since GHSs are known to compliment GHRH actions, the possibility of GHSs influencing feeding behaviour has therefore been examined, since GHRH affects appetite (Frohman & Jansson, 1986). Picomolar doses of i.c.v KP-102 stimulate feeding in mammals (Locke et al., 1995; Dickson et al., 1995; Ghigo et al., 1997; Locatelli et al., 1997; Svensson & Bergtsson, 1999). Moreover, when GHSs are co-administered with GHRH the food intake is amplified; again demonstrating a synergism between GHRH and GHSs at central sites (Okada et al., 1996).

5.2 Sleeping patterns

Acute i.v GHRP-6 and oral MK-677 administration promotes SWS and stage 2 and 4 sleep (non-REM sleep) in normal male subjects, whilst MK-677 increased REM sleep too (Steiger, 1999). Therefore, the natural GHS-R ligand is involved in the somatotrophic system that participates in sleep regulation (Frieboes et al., 1995; Copinschi et al., 1997; Frieboes et al., 1999). GHSs could, therefore, be used as a form of treatment

for sleep disorders since they are better related to the normal physiological components of sleep compared to benzodiazepines, which are currently used to suppress SWS, EEG-delta waves and REM sleep (Steiger, 1999).

5.3 Learning & memory

Roles for GHSs unrelated to GH release have been proposed because of the presence of the GHS-R in the cerebellum and cerebral cortex (Bennett et al., 1997; Yokote et al., 1998; Smith, 1998). These receptors are thought to mediate actions of GHSs on learning and memory (Ghigo et al., 1999; Smith et al., 1999a). GHSs have also been implicated in reinforcement behaviour (Walker & Bercu, 1998; Smith et al., 1999a).

Taken together, the presence of specific GHS-Rs in the pituitary and the CNS suggest that the endogenous GHS-R ligand has roles in both neural and neuroendocrine function.

E. Clinical perspectives

Children with idiopathic short stature, familial short stature, intra-uterine growth retardation (IUGR), Turner's syndrome and chronic renal failure respond to acute GHS treatment when administered orally or intranasally, with no undesirable effects (Smith & Brook, 1988; Bowers et al., 1991; Lanes, 1995; Laron et al., 1995; Ghigo et al., 1998; Bach, 1998; Casanueva & Dieguez, 1999; Guzzaloni et al., 1999; Laron, 1999). GHSs increase GH and growth velocity in children, but effects of GHSs on bone mass in adults is uncertain. In adult rats, however, Ipamorelin significantly increased GH secretion and cortical bone mass (Svensson & Bentsson, 1999). In human adults, GHRH agonists and GHS analogs have been suggested to have potential for treating hypopituitarism (Goth et al., 1992), catabolic states (Van der Berghe et al., 1996), wound healing and osteoporosis (Table 1.4). (Dahms et al., 1989; Schally & Comaru-Schally, 1998; Van der Berghe et al., 1999a,b). In a number of conditions, such as anorexia nervosa, diet-induced catabolism and critical illness, tissue catabolism is driven by poor nutritional intake or periods of complete fasting. Fasting results in low plasma IGF-1 and high GH in many catabolic

Table 1.4: Clinical uses of GH-secretagogues (GHSs)

Clinical Condition	Type of Secretagogue	Dose (i.v.)	Efficiency of GH secretion
Anorexia	Hexarelin	1 ug/kg	***
Critical illness	GHRH	1 ug/kg	*
	GHRP-2	1 ug/kg	***
	GHRP-2 + GHRH	1 ug/kg	*****
Cushing's Syndrome	GHRH	100 ug/kg	---
	GHRP-2	1 ug/kg	*
	GHRP-2 + GHRH	1 ug/kg	**
Diet-induced catabolism	Hexarelin	1 ug/kg	***
Hyperthyroidism	GHRH	100 ug/kg	---
	GHRP-2	1 ug/kg	***
	GHRP-2 + GHRH	1 ug/kg	***
Renal failure	Hexarelin	1 ug/kg	**

- * (slight increase in GH secretion)
- ** (moderate increase in GH secretion)
- *** (great increase in GH secretion)
- ***** (exceptional increase in GH secretion)
- (no change in GH secretion)

(Adapted from Jenkins & Ross, 1999).

states in humans (Van der Berghe, 1999b; Jenkins & Ross, 1999). Exogenous GH has been shown to be useful for treating some catabolic states by increasing food intake; however, it has been suggested that GHSs may offer a convenient alternative form of treatment in those patients who are GH resistant, since GHS actions on food intake can be independent of GH (Jenkins & Ross, 1999). Furthermore, the blunted pattern of GH release present in the chronic phase of critical illness can be reversed by the use of GHSs (van der Berghe, 1999). However, the potential utility of GHSs as agents to treat selected catabolic states requires long-term studies with clinical end-points.

VI. GHRELIN

A. Introduction

Since the GH-releasing activities of the GHRPs/GHSs are distinct from the GH-releasing activity of GHRH, it was thought that they mimicked an unknown endogenous factor (Bowers, 1996, 1999). This belief has now been confirmed, since an endogenous ligand for the GHS-R has been identified. This ligand, ghrelin (*ghre* = “grow” Proto-Indo-European root), was initially isolated from the rat and human stomach (Kojima et al., 1999), although it was subsequently shown to be present in the brain and pituitary gland (Kamegai et al., 2000; Hosoda, 2000; Korbonits et al., 2001a,b).

Kojima et al., (1999) constructed a stable CHO cell line expressing the rat GHS-R (CHO-GHSR62) to identify the endogenous ligand, by monitoring changes in induced intracellular calcium concentrations $[Ca^{2+}]_i$. Extracts were taken from a range of tissues and the highest activity was found in the stomach extract. The stomach was therefore purified by chromatography, high performance liquid chromatography (HPLC) and reverse-phase (RP)-HPLC (RP-HPLC) (Kojima et al., 1999). The ligand was termed ghrelin and found to be a 28 amino-acid peptide that specifically activates the GHS-R to induce the release of GH with great potency *in vitro* and *in vivo* (Kojima et al., 1999). Furthermore, the presence of ghrelin in both human and rat strengthens the hypothesis that this ligand is highly conserved between species (Smith et al., 1999a; Palyha et al., 2000). Thus, both GHRH and ghrelin, in mammals at least, are thought to participate in

pituitary GH regulation (Ghigo et al., 1997; Smith, 1998; Camanni et al., 1998; Kojima et al., 1999). The possible role of ghrelin as a GRF in birds has yet to be determined.

B. Structure

Protein sequencing and cDNA analysis established that the amino acid sequence of rat ghrelin is: GSXFLSPEHQKAQQRKESKKPPAKLQPR, where the third residue X was later revealed to be serine (Kojima et al., 1999). However, synthetic ghrelin (X=serine) did not increase $[Ca^{2+}]_i$ in CHO-GHSR62 cells, indicating that serine 3 must be modified in naturally occurring ghrelin. Serine 3 was later demonstrated to be octanoylated, as the hydrogen atom of the hydroxyl group in serine 3 is replaced by a $C_7H_{15}CO$ moiety. Rat cDNA was used to screen a human stomach cDNA library and it was discovered that rat and human ghrelin sequences differ by only 2 amino acids (Figure 1.2). This high degree of sequence homology indicates ghrelin is highly conserved. Purification of human ghrelin from the stomach also revealed a modified serine 3, as found in rat ghrelin.

It is interesting to note that no structural homology exists between ghrelin and any synthetic GHS (Matsumoto et al., 2001), yet ghrelin is the endogenous ligand for the GHS-R. However, ghrelin is not the sole ligand for the GHS-R, as a second ligand was purified and characterized from rat stomach (Hosoda et al., 2000a,b). This second ligand is identical to ghrelin, with an octanoylated serine 3, except for the deletion of a glutamine at position 14 of the peptide. This peptide has been termed des-Gln¹⁴-ghrelin. Prepro-des-Gln¹⁴-ghrelin was also cloned and is identical to prepro-ghrelin, except for a missing CAG nucleotide between position 134 and 135 in prepro-des-Gln¹⁴-ghrelin. It is thought that the ghrelin gene undergoes alternative splicing to give rise to the two mature peptides. The 3' end of an intron present between the coding sequence for Gln¹³ and Gln¹⁴ of the ghrelin sequence has two tandem CAG sequences that act as the splice acceptor sites that either produces ghrelin or des-Gln¹⁴-ghrelin (Hosoda et al., 2000a,b; Kaji et al., 2001).

Recent evidence has indicated structural- and functional-related similarities between ghrelin and other hormones isolated from the gastrointestinal tract, particularly,

*



1 10 20

G S S F L S P E H Q K A Q Q R K E S K K P P A K L Q P R

G S S F L S P E H Q R V Q Q R K E S K K P P A K L Q P R

1 10 20



*

Figure 1.2: A comparison between the structure of rat and human ghrelin. One letter amino acid notation is used to illustrate rat ghrelin sequence (top) and human ghrelin sequence (bottom). The highlighted amino acids (grey boxes) at positions 11 and 12 of both rat and human ghrelin sequences indicate these peptides only differ by 2 amino acids. The asterisks indicate a *n*-octanoyl modification at serine 3 in both peptides.

motilin. Human ghrelin and human motilin possess 30% sequence homology, and their respective receptors are 50% identical (Asakawa et al., 2001). However, ghrelin and motilin-related peptide (MTLRP) have 100% sequence homology (Folwaczny et al., 2001). They are, therefore, the same peptide (Figure 1.3). This confusion occurred because rat and human ghrelin (Kojima et al., 1999) and mouse MTLRP (Tomasetto et al., 2000) sequences were submitted almost simultaneously (1 day apart) into the databases (accession no.s AB029433 and AB029434 for rat and human ghrelin sequences and AJ243503 for mouse MTLRP). Furthermore, both groups of researchers had different objectives, since Kojima and co-workers were specifically trying to isolate the GHS-R ligand whilst Tomasetto and co-workers were on the search for new proteins that are restricted to the gastric epithelium. Therefore, the same peptide was termed (ghre = 'to grow') due to its potent GH-releasing activity, and, MTLRP due to its sequence similarity with motilin. The only difference between the ghrelin and MTLRP sequences reported was that Tomasetto et al., (2000) did not find the n-octanoyl modification at serine 3 in MTLRP. This may be explained by the different methodologies used by the two groups of researchers (Rincon et al., 2001), since Tomasetto et al., (2000) expressed the peptide in transfected COS-1 cells that were not able to perform the octanoylation.

C. Ghrelin gene structure

The genomic organization of the ghrelin gene has been analyzed in mice and rats (Tanaka et al., 2001) and in humans (Wajnrajch et al., 2000). The mouse/rat ghrelin gene consists of 5 exons and 4 introns with the entire cDNA sequence being 3748 base pairs (bp) in length (accession no. AB060078, GenBank databases). The human ghrelin gene consists of 5 exons and 3 introns with the cDNA sequence being 5199 bp long (accession no. AF296558, Genbank databases). The exon/intron organization of the human ghrelin gene is shown in Figure 1.4.

Human prepro-ghrelin	M P S P G T V C S S L L L G M L W L D L A M A	signal sequence
Human prepro-MTLRP	M P S P G T V C S I I I I G M I W L D L A M A	signal sequence
Human prepro-ghrelin	G S S F L S P E H Q R V Q Q R K E S K K P P A	1-23
Human prepro-MTLRP	G S S F L S P E H Q R V Q Q R K E S K K P P A	1-23
Human prepro-ghrelin	K L Q P R A L A G W L R P E D G G Q A E G A E	24-46
Human prepro-MTLRP	K L Q P R A L A G W L R P E D G G Q A E G A E	24-46
Human prepro-ghrelin	D E L E V R F N A P F D V G I K L S G V Q Y Q	47-69
Human prepro-MTLRP	D E L E V R F N A P F D V G I K L S G V Q Y Q	47-69
Human prepro-ghrelin	Q H S Q A L G K F L Q D I L W E E A K E A P A	70-92
Human prepro-MTLRP	Q H S Q A L G K F L Q D I L W E E A K E A P A	70-92
Human prepro-ghrelin	D K	93-94
Human prepro-MTLRP	D K	93-94

Figure 1.3: Protein sequences of human prepro-ghrelin and human prepro-motilin-related peptide (MTLRP). One letter amino acid notation is used and are numbered on the left. The sequences of the mature peptides are highlighted (grey boxes) and the black circle represents serine 3 to be modified (octanoylated) in human prepro-ghrelin.

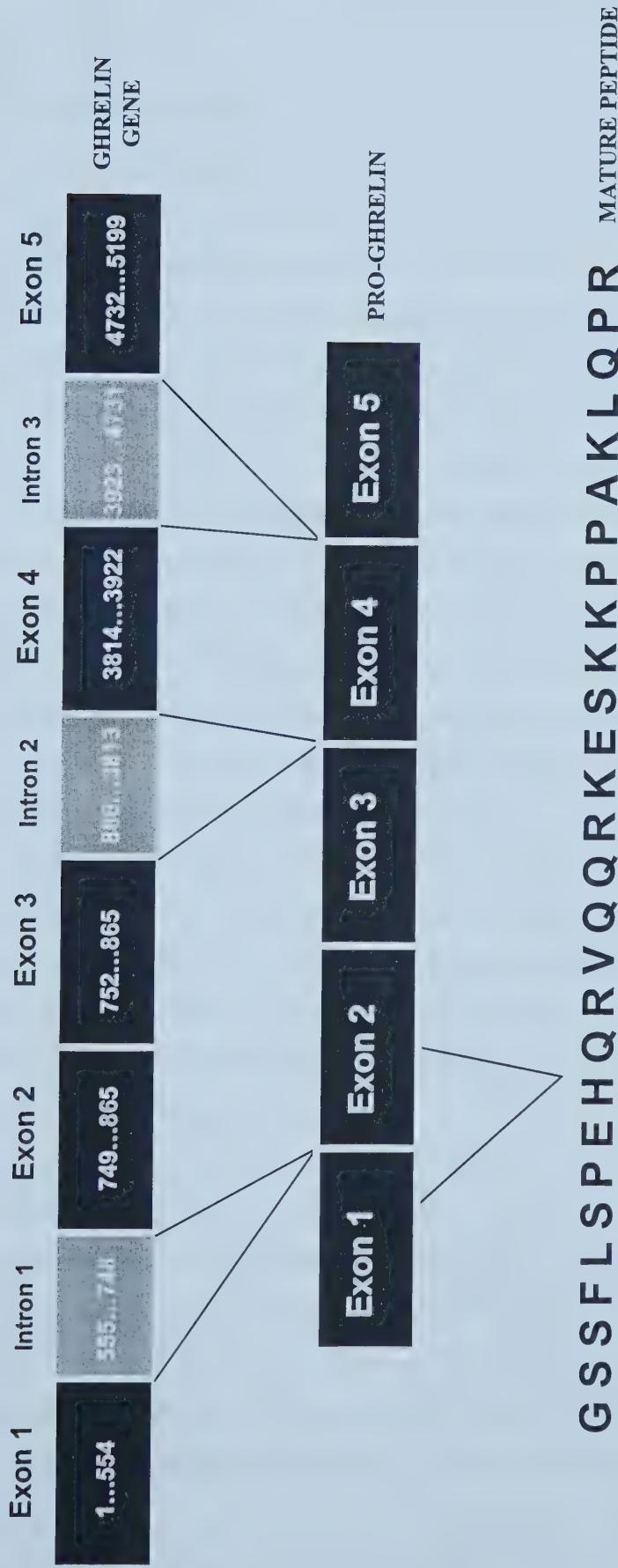


Figure 1.4: A schematic of human ghrelin gene. Exons and introns are shown by boxes (black and grey boxes respectively). Base pairs are indicated below each exon and intron respectively. Octanoylation occurs between 522...524 base pairs and alternative splicing of the gene occurs between 752...865 bp (exon 3) which gives rise to des-Gln¹⁴-ghrelin. The sequence of the gene can be found in GenBank/EMBL/DDBJ data bases under the accession No. AF296558

D. Distribution

1. Stomach/Gastrointestinal tract

As a GH-releasing factor, ghrelin might be expected to be most abundant in the hypothalamus. It is, however, most abundant in the stomach. Using RIA, ICC, *in situ* hybridization and RT-PCR, ghrelin and des-Gln¹⁴-ghrelin were demonstrated in the fundus of the stomach, where endocrine cells containing ghrelin are expressed from the neck to the base of the rat and human oxytic gland (Kojima et al., 1999; Date et al., 2000b; Gualillo et al., 2001b). Smaller amounts of both ghrelin were also found in the duodenum, jejunum, ileum and colon of the G.I. tract (Date et al., 2000a,b; Hosoda et al., 2000b). Within the oxytic mucosa, ghrelin was found in X/A-like endocrine cells which are now also designated as ghrelin cells (Gr cells) (Dornoville et al., 2001). Immunogold staining and immunoelectron microscopy revealed Gr cells to be round to ovoid in shape with electron dense granules (approx. 120±30nm diameter). These cells are located in close association with the capillary network of the stomach with no contact with the lumen; thus, the Gr cells either respond to physical stimuli from the lumen or chemical signals from the basolateral site (Date et al., 2000a, b).

It is thought that ghrelin is secreted from the stomach into the bloodstream, and circulates around the body to reach the pituitary and hypothalamus, and possibly other target tissues (see Table 1.3 for GHS-R distribution). Ghrelin's stimulatory action on GH release and food intake may be regulated partly by a negative feedback mechanism, which is likely to involve SRIF and anorexigenic peptides respectively, as well as a down regulation of GHS-R binding sites.

2. Hypothalamus

Ghrelin-containing neurons have been located within a small number of neurons in the arcuate nucleus (ARC) of the hypothalamus (Kojima et al., 1999). The ARC is also the site in which other hormones involved in GH regulation and the regulation of feeding (such as GHRH, AGRP and NPY) (Hewson & Dickson, 2000). Since GHS-R is expressed in the hypothalamus and pituitary, it is thought that ghrelin from the ARC can

stimulate the hypothalamus and/or hypophysis directly or be transported to the pituitary to stimulate GH release.

3. Pituitary gland

Ghrelin mRNA and peptide are expressed in the pituitary gland of normal healthy human subjects. No age-variation was seen, as ghrelin mRNA was present in subjects ranging from 1 day to 54 years of age (Korbonits et al., 2001a, b). Therefore, locally-produced ghrelin could have autocrine/paracrine actions on GH release. It was recently demonstrated that GHRH increases the expression of ghrelin in the pituitary gland of the rat (Kamegai et al., 2001). Ghrelin-immunoreactivity was also found in adenomatous human pituitaries, particularly in gondotroph and lactotroph adenomas, with weaker staining in somatotroph, corticotroph and non-functioning adenomas (Korbonits et al., 2001a).

4. Other tissues

In addition to the gastrointestinal tract and the brain, prepro-ghrelin gene is expressed in the mouse kidney and in mesangial cells and podocytes of the glomerulus (Mori et al., 2000). Structural analysis of renal ghrelin suggests it is acylated as in the stomach (Mori et al., 2000). The function of ghrelin in the kidney and glomerulus is, however, unknown. Interestingly, ghrelin mRNA and peptide have also been localized in rat and human placentae (Gualillo et al., 2001a). In rats, ghrelin was localized within the labyrinthine trophoblast in late gestation stages, whilst in humans ghrelin was found in cytotrophoblast and syncytiotrophoblast cells in the first trimester of pregnancy (Gualillo et al., 2001a). It may therefore be predicted that the placenta is a site of GH gene expression. The distribution of ghrelin is summarized in Table 1.5.

Table 1.5: *Tissue distribution of the ghrelin peptide.*

Tissue	Species	References
<i>Gastrointestinal tract</i>		
Stomach	Rat Human	Kojima et al., 1999 Date et al., 2000a,b
Duodenum	Human	Hosoda et al., 2000b
Jejunum	Human	Date et al., 2000a,b
Ileum	Human	Date et al., 2000a,b
Colon	Human	Hosoda et al., 2000b Date et al., 2000a,b
<i>Brain</i>		
Hypothalamus	Mouse Rat Human	Hosoda, 2000 Kojima et al., 1999 Korbonits et al., 2001a
Pituitary gland	Human	Korbonits et al., 2001a,b
<i>Peripheral tissues</i>		
Kidney	Mouse	Mori et al., 2000
<i>Other</i>		
Placenta	Rat Human	Gualillo et al., 2001a Gualillo et al., 2001a
Fetus	Sheep	Roelfsema, 2001
<i>Tumours/Adenomas</i>		
Somatotroph adenomas	Human	Korbonits et al., 2001a
Coticotroph adenomas	Human	Korbonits et al., 2001a
Gonadotroph adenomas	Human	Korbonits et al., 2001a
Lactotroph adenomas	Human	Korbonits et al., 2001a

E. GHS-R/Ghrelin-R

Since ghrelin is the natural endogenous ligand for the GHS-R, this receptor is now termed the ghrelin-receptor (ghrelin-R). The activation of this receptor in the pituitary is due to the first 18 amino acids of the peptide. This truncated peptide does not, however, have any hypothalamic action *in vivo* (Tolle et al., 2001) probably because the last 10 amino acids of the C terminal sequence are thought to be required for ghrelin to cross the blood-brain barrier (Tolle et al., 2001).

Recently, ghrelin and the second generation GHSs (MK-677 & GHRP-6) have been found to occupy different binding pockets of the GHS-R, than those of the first generation of GHSs (Sun et al., 2001). Hence, the second generation GHSs mirror the biological actions of ghrelin more closely than the other GHS groups. However, a number of GHS-R sub-types may exist. Indeed, Howard et al., (1996) has identified a GHS-R1 and postulated the existence of at least a second sub-type: GHS-R2. Knock-out mice of the cloned GHS-R1 still exhibit normal growth, providing evidence for other receptor sub-types that still allow ghrelin to mediate its effects (Dieguez & Casanueva, 2000). It is now also speculated that if GHS-R sub-types exist, ligands (U-factor/s) other than ghrelin and des-Gln¹⁴-ghrelin may also exist (Bowers, 1999). Ahnfelt-Ronne et al., (2001) hypothesized GHSs (NN-703 and ipamorelin/NN-161) act as ghrelin secretagogues and may mimic the actions of other endogenous ligands that function to regulate ghrelin synthesis and, indirectly, GH release.

F. Ghrelin regulation/secretion

The factors regulating the synthesis and secretion of ghrelin are far from clear. However, a physiological role for ghrelin in feeding behaviour has been indicated by upregulation of its expression in the rat stomach during fasting (Toshinai et al., 2001). Plasma ghrelin levels are also increased during fasting in rats (Toshinai et al., 2001) and in cows and goats (Hayashida et al., 2001). Increased ghrelin secretion from the stomach during fasting would be likely to stimulate feeding, in order to maintain a positive energy balance (Toshinai et al., 2001).

Indeed, ghrelin induces food intake and an increase in body weight in rats (Tschoop et al., 2000). Insulin-induced hypoglycemia and administration of leptin also cause upregulation of ghrelin expression in the stomach of rats (Toshinai et al., 2001). Both insulin and leptin are known to regulate energy homeostasis by reducing food intake, whilst ghrelin functions to increase food intake during periods of negative energy balance (Toshinai et al., 2001). Not surprisingly, ghrelin secretion is, therefore, reduced upon consumption of food, when energy requirements have been met (Toshinai et al., 2001). The decrease in circulating ghrelin are specifically due to ingested nutrients and not simply due to stomach expansion, since oral administration of water did not alter serum ghrelin but ghrelin increased after glucose administration (Tschoop et al., 2000). A physiological role for ghrelin in initiating food intake has also been demonstrated in humans (Cummings et al., 2001). Human plasma ghrelin concentrations rise nearly twofold before a meal and rapidly decrease 1 hour following food consumption. Plasma insulin and leptin are found to be inversely related to plasma ghrelin, suggesting reciprocal actions between insulin and leptin with ghrelin in energy homeostasis (Cummings et al., 2001). The regulation of ghrelin secretion, as well as the biological effects of ghrelin, therefore appear to be antagonistic to those of leptin (Horvath et al., 2001).

Changes in ghrelin levels have been associated with the pathophysiology of obesity. Surprisingly, plasma ghrelin levels are markedly decreased in obese subjects compared with age-matched lean control subjects (Tschoop et al., 2001). This reflects downregulation of ghrelin as a result of positive energy balance accompanied by obesity. Furthermore, mutations of the ghrelin gene have also been associated with obesity (Ukkola et al., 2001). Using 96 obese subjects and 96 non-obese subjects, two mutations of the preproghrelin gene were observed in the overweight patients, which result in a defective peptide. One mutation occurs at Arg51Gln (this site corresponds to the last amino acid of the mature ghrelin product), and the second mutation occurs at Leu72Met of the preproghrelin gene in obese subjects (Ukkola et al., 2001). Thus, mutations of the ghrelin gene are thought to be involved in the etiology of human obesity.

G. Ghrelin actions in mammals

1. GH regulation

A large body of evidence indicates ghrelin is a potent GH-releasing factor in mammals (Kojima et al., 1999; Wren et al., 2000; Takaya et al., 2000; Date et al., 2000a; Seone et al., 2000; Bowers, 2001; Arvat et al., 2001). It is thought that ghrelin, like GHSs, has two sites of action for GH regulation: the pituitary and hypothalamus. Ghrelin, when administered i.p (30nmol), i.c.v (2nmol) or i.v (5ug/kg) to rats has been shown to increase GH release in a dose-dependent manner. The maximal GH response is observed within 5-15 minutes and returns to basal level at 60 minutes (Wren et al., 2000; Takaya et al., 2000; Date et al., 2000a). Repeated administration of ghrelin over a short period of time results in homologous desensitization, as observed with GHSs (Bowers, 2001). Ghrelin administered every 3-4 hours does not, however, result in desensitization. This is due to an increase in the amount of GH present in releasable pools in the pituitary gland (Tolle et al., 2001). Ghrelin is, thus, likely to be involved in GH synthesis as well as GH release. This possibility is supported by the recent discovery that ghrelin increases pituitary-specific transcription factor-1 (pit-1) expression in rats (Garcia et al., 2001). Ghrelin may also promote GH secretion by actions at the hypothalamic level that inhibit SRIF release (Tolle et al., 2001) and stimulate GHRH release (Dickson, 1999; Tannenbaum & Bowers, 2001; Hataya et al., 2001).

In humans, ghrelin and GHRH have comparable potencies, with EC₅₀ values of 2.1×10^{-9} M and 0.6×10^{-9} M respectively (Kojima et al., 1999). Des-Gln¹⁴-ghrelin is also equipotent to ghrelin in stimulating GH release (Hosoda et al., 2000). In rats, ghrelin has been demonstrated to be 2-3 x more potent than GHRH *in vivo* but GHRH is more potent *in vitro* (Seoane et al., 2000). GHRH and ghrelin are thought to stimulate GH release from the pituitary through different mechanisms and thus they have synergistic or at least additive effects when present together (Date et al., 2000a; Seoane et al., 2000).

Although GHRH specifically regulates GH secretion, some studies in rats have reported that ghrelin increases ACTH and cortisol release (as induced by GHSs), with no effects on the release of luteinizing hormone (LH), thyrotropin-stimulating hormone (TSH) or prolactin (PRL) (Wren et al., 2000). A slight increase in PRL levels was,

however, observed in humans following ghrelin treatment (Takaya et al., 2000). These effects could be species-dependent.

3. Feeding regulation

Ghrelin, like GHRH, is also thought to be involved in feeding behaviour and energy balance (Wren et al., 2000; Hewson & Dickson, 2000; Kamegai et al., 2000; Shintani et al., 2001; Tschop et al., 2000, 2001; Toshinai et al., 2001). A single i.c.v injection of ghrelin rapidly stimulates increased food intake in a dose-dependent manner in fasted rats, with the effect sustained for up to 24 hrs. Ghrelin, therefore, functions in response to a negative energy balance by increasing nutrient intake to increase energy levels. This would, in the longer turn, act to counteract tissue catabolism. Furthermore, ghrelin-induced feeding in the rat is seen not only during the dark phase (feeding) but also during the light phase (satiated) (Wren et al., 2000; Nakazato et al., 2001). Both ghrelin and GHS-R/ghrelin-R have been localized in the ARC (site involved in food and body weight regulation) and the central administration of ghrelin induces c-fos and erg-1 (early growth response factor-1) proteins, indicating the ARC is an important site of ghrelin action (Hewson & Dickson, 2000).

Other neuropeptides regulating food intake, such as neuropeptide Y (NPY) and agouti-related peptide (AGRP), are also localized within the ARC. It is therefore postulated that ghrelin induces increased food consumption through stimulation of these other orexigenic peptides (Figure 1.5) (Kamegai et al., 2000; Shintani et al., 2001). Using *in situ* hybridization it was shown that ghrelin increases AGRP mRNA levels in the hypothalamus, which in turn stimulates feeding (Kamegai et al., 2000), but has no effect on NPY levels, since ghrelin stimulates food intake in NPY deficient mice (Tschop et al., 2000). Furthermore, ghrelin's action on feeding behaviour also occurs through a GH-independent pathway, as GH-deficient rats gained weight when stimulated with ghrelin centrally (Wren et al., 2000; Tschoop et al., 2000; Nakazato et al., 2001; Shintani et al., 2001).

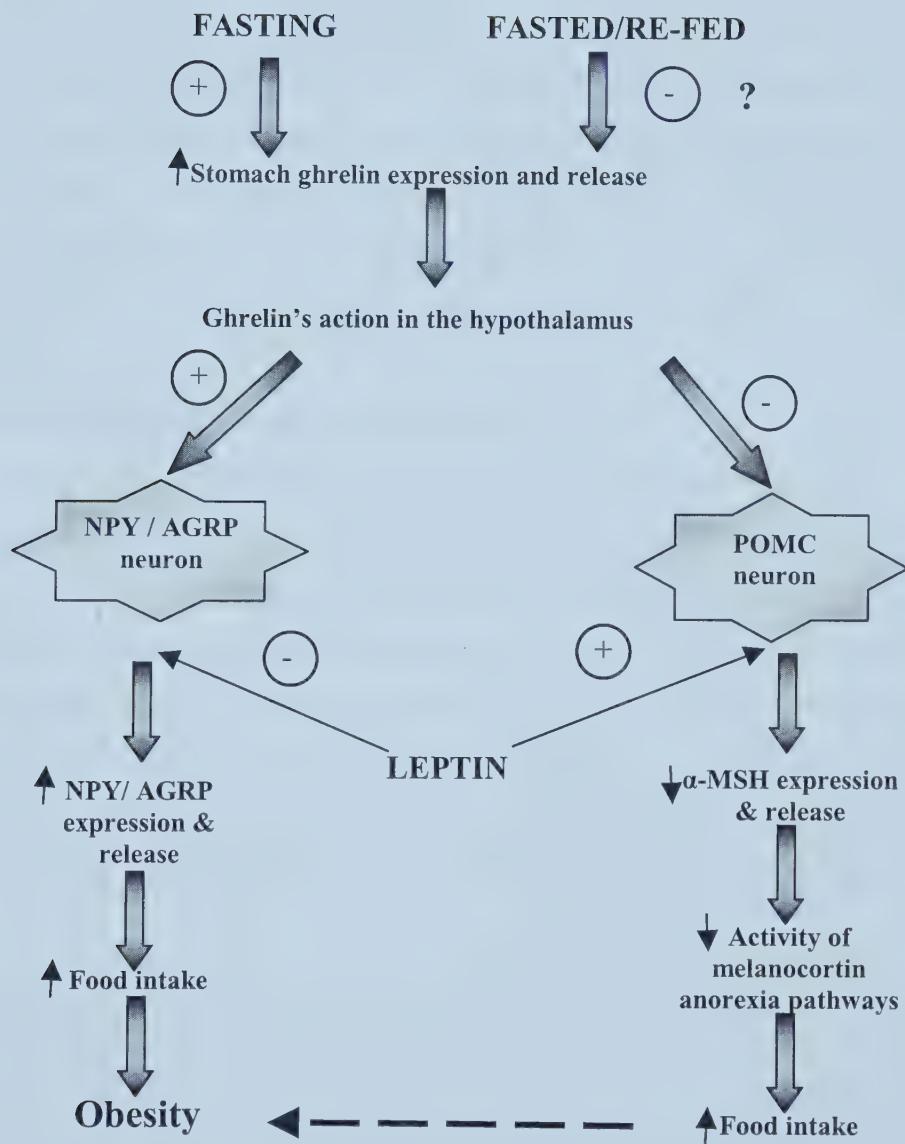


Figure 1.5: A schematic of ghrelin's proposed role in feeding behaviour.

During fasting stomach ghrelin expression increases and ghrelin is therefore released into the circulatory system. Ghrelin travels via the bloodstream to activate NPY (neuropeptide Y) and AGRP (agouti-related peptide) neurons via the GHS-R present on these hypothalamic nuclei to cause NPY/AGRP release. NPY/AGR, then in turn, increase food intake to provide a positive energy balance. Conversely, stomach ghrelin expression decreases after re-feeding. This decreases the inhibitory activity of POMC (pro-opiomelanocortin) on feeding. The activity of anorexigenic pathways is decreased and therefore results in food intake. Leptin, a satiety signal, is thought to be reciprocally controlled to ghrelin and therefore acts to decrease NPY/AGRP stimulatory action on feeding whilst it stimulates POMC to inhibit food intake. It is yet to be established what role central vs. peripheral ghrelin plays in feeding regulation and at which sites ghrelin interacts with leptin.

Ghrelin uses the hypothalamic NPY/Y1-R pathway for its orexigenic effects on food intake, since NPY-R antagonist (Y1-R antagonist) completely abolished the effects of ghrelin when co-administered centrally (Shintani et al., 2001). Similarly, the satiety effect of leptin was abolished when co-injected with ghrelin; thus, indicating an antagonistic relationship between ghrelin and leptin in the control of feeding behaviour (Toshinai et al., 2001). Ghrelin mRNA levels within the stomach were shown to increase upon fasting or leptin administration and decreased upon re-feeding, thus ghrelin is thought to signal when an increase in energy intake (Tschop et al., 2000).

4. Gastrointestinal tract: Function and regulation

Since the stomach is the principal site of ghrelin synthesis and secretion, it is not surprising that ghrelin is thought to be also involved in the regulation of gastric acid secretion and gastric motility (Masuda et al., 2000; Date et al., 2001). Other neuropeptides that regulate feeding behaviour also play a part in the central regulation of G.I. tract function (Masuda et al., 2000). Centrally (i.c.v) or systemically administered ghrelin increases gastric acid secretion and gastric motility in a dose-dependent manner in rats (Masuda et al., 2000), via the vagal nerve, the nucleus of solitary tract (NTS) and the dorsolateral nucleus of vagus (DMNV) within the medulla oblongata, via the GHS-R (Date et al., 2001).

5. Hemodynamic effects

Hemodynamic effects of ghrelin were investigated due to its potent GH-releasing abilities and the fact that GH is involved in myocardial growth and cardiac function (Nagaya et al., 2001). The presence of GHS-R/ghrelin-R in the myocardium and aorta suggests ghrelin has direct actions on the heart by decreasing mean arterial pressure and increasing cardiac output without altering heart rate (Nagaya et al., 2001). Thus, ghrelin has possible beneficial hemodynamic effects for patients suffering with congestive/chronic heart failure, as it induces a long-lasting hypotensive action by decreasing cardiac overload (Nagaya et al., 2001).

H. Ghrelin actions in birds

1. A GRF?

No studies are currently available on the possible GH-releasing abilities of ghrelin in birds, despite the recent cloning and characterization of the GHS-R (ghrelin being the natural ligand) on chicken pituitary membranes (Toogood et al., 1999; Gaylinn et al., 2000) and the potent GH-releasing abilities of the artificial GHS, L-692, 429 and L-163, 255, in chickens (Geris et al., 1998a, 2001).

2. An orexigenic factor?

A recent study investigated the effects of ghrelin and GHRH on food intake in neonatal chicks, since a wealth of information is now available indicating the potent actions of ghrelin as a feeding stimulant in mammalian species (Wren et al., 2000; Hewson & Dickson, 2000; Shintani et al., 2001). Thus, the possibility that ghrelin may have similar effects in chickens was investigated. Day-old male broiler chicks received i.c.v injections of both rat ghrelin and human GHRH, at doses of 0.25, 0.5 and 1.0 nmol. Both neuropeptides potently suppressed food intake in a dose-dependent manner, as measured by the disappearance of food from the feeder and decreased body weight (Furuse et al., 2001). It was therefore postulated that the feeding mechanisms of chicks differ from those in mammals (Furuse et al., 2001). Overall, it was concluded that mammalian ghrelin and GHRH exhibit biological effects in young chicks. This study also provided support for the presence of mammalian-like GHRH and ghrelin receptors and their respective ligands within the avian brain (Furuse et al., 2001).

VII. EXPERIMENTAL RATIONALE

Many reports have appeared on the presence of ghrelin, the natural ligand for the GHS-R, in the stomach and brain of mammalian species. In mammals, ghrelin is primarily involved in GH regulation, having interactions with both GHRH and SRIF to stimulate GH release. The dual GHRH-SRIF concept of GH regulation has therefore been expanded in mammals to include a third (ghrelin) regulatory system.

At present, studies on the presence and actions of ghrelin in GH regulation have only been investigated in mammals. Therefore, the possibility of a ghrelin-like peptide being present and participating in regulating GH secretion in non-mammalian species remains to be elucidated.

The recent cloning and characterization of the GHS-R (ghrelin being the natural ligand) on chicken pituitary membranes (Toogood et al., 1999; Gaylinn et al., 2000), suggests the involvement of a ghrelin-like peptide in the GH regulation in avian species. This is further strengthened by the potent GH-releasing abilities of artificial GHSs, L-692, 429 and L- 163, 255, which mimic the actions of ghrelin, in chickens (Geris et al., 1998a; 2001). The possibility that a ghrelin-like peptide is present in the avian system, and that it is involved in GH regulation in the domestic fowl (*Gallus domesticus*), was therefore examined in this thesis.

VIII. HYPOTHESIS

The hypothesis of this study is that ghrelin is present in the avian hypothalamus, and is a GH-releasing factor in birds.

IX. SPECIFIC AIMS

The specific aims of the thesis were:

- 1) To determine the presence of ghrelin in the chicken
- 2) To determine if ghrelin induces GH release in the chicken

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CHAPTER TWO

Ghrelin: A hypothalamic growth hormone-releasing factor in the domestic fowl (*Gallus domesticus*).



Some of the results in this chapter were presented at the 34th International Congress of Physiological Sciences. Ahmed, S and Harvey, S (2001) Ghrelin: A Growth Hormone-Releasing Factor in Birds. Christchurch, New Zealand (Abstract 1453). Data from this chapter were also published in Ahmed, S and Harvey, S (2001) Ghrelin: A Growth Hormone-Releasing Factor in Birds. *Journal of Endocrinology*, (In Press).

1. INTRODUCTION

Growth hormone (GH) secretion is regulated by a myriad of interacting, multihierarchical factors that dynamically modify somatotroph function at neural and humeral interfaces in neurocrine, neuroendocrine, endocrine and paracrine ways (Harvey, 1995). The hypothalamus is, however, thought to primarily regulate GH release in mammals through antagonistic interactions between stimulatory (GH-releasing hormone, GHRH) and inhibitory (somatostatin, SRIF) hypophysiotropic releasing hormones. A third hypothalamic factor, ghrelin, may also be involved (Dieguez & Casanueva, 2000; Kojima et al., 2001), since it is present in the arcuate nucleus (ARC) of the mouse, rat and human hypothalamus (Kojima et al., 1999; Hosoda, 2000; Korbonits et al., 2001) and is 3-5 more potent than GHRH in stimulating GH release in these species (Kojima et al., 1999; Arvat et al., 2000; Seoane et al., 2000). Ghrelin mediates its actions on GH release via distinct receptors termed GH-secretagogue receptors (GHS-R) in mammals (Howard et al., 1996; Muccioli et al., 1997). It is, however, unknown if ghrelin is involved in GH regulation in other species.

In birds, many factors influence the release of GH from the pituitary gland. Thyrotropin-releasing hormone (TRH) is a physiologically important GH-releasing factor (GRF) in birds (Harvey, 1990), and mammalian GHRH peptides have also been shown to induce GH secretion in avian species (Harvey & Scanes, 1984; Foltzer-Jourdainne et al., 1988; Moellers & Cogburn, 1994). Although the presence of a chicken GHRH (cGHRH) gene has been reported in the chicken brain (McRory et al., 1997) and the GHRH-R has been cloned and characterized in chickens (Toogood et al., 1999), chicken GHRH has minimal GH-releasing activity in chickens (Harvey, 1999). The possibility that ghrelin may participate in GH release in birds was therefore assessed, especially as ghrelin is thought to be the endogenous ligand for a GH-secretagogue (GHS) receptor identified in chicken pituitary glands (Toogood et al., 1999; Gaylinn et al., 2000).

2. MATERIALS & METHODS

Tissues

White Leghorn chickens at 4 week of age were killed by cervical dislocation (with approval from the University of Alberta Health Sciences Animal Welfare Committee) and hypothalami were rapidly dissected from the heads, washed in ice-cold phosphate buffered saline (PBS, pH 7.4) and collected into freshly prepared paraformaldehyde (4% w/v) (Sigma, Mississauga, Ontario, Canada). The hypothalami were removed in one piece, with two parallel cuts 2.0-2.5mm lateral to the midline, a transverse cut in front of the preoptic area and the roots of the oculomotor nerves and a cut 4-5mm deep parallel to the base of the brain (MacNamee & Sharp, 1989).

The rat stomach was used as a positive control for the detection of ghrelin immunoreactivity, since ghrelin is abundantly found in the mammalian stomach (Kojima et al., 1999; Date et al., 2000a). Sprague-Dawley male rats, weighing approximately 250-300 g (University of Alberta Animal Services), were anesthetized, killed by cervical dislocation and the stomachs were rapidly removed and washed in ice-cold PBS. For a comparison, the chicken stomach and the gastrointestinal tract (the crop, ileum, duodenum and colon) were also dissected and washed in ice-cold PBS.

Immunocytochemistry

All tissues were fixed in freshly prepared paraformaldehyde (4% w/v) (Sigma, Mississauga, Ontario, Canada) and left to fix overnight at 4°C. Tissues were then dehydrated in a graded series of alcohol (50% v/v, 15-30 min; 70%, 30-60 min; 95%, 30-60 min; 100%, 60-120 min) and cleared with hemo-de (a de-paraffinizing agent) (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax for 24 h at 60 °C, under normal atmospheric pressure. Serial transverse sections (4-8 μ m) were cut with a microtome and mounted onto treated slides (Fisher Scientific). The sections were cut anterior-dorsally, using coordinates A10.0 to A5.6,

according to the histological atlas of Kuenzel & Masson (1988). Magnocellular nuclei were identified according to Yasuda (1980) and Mikami (1985).

Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlington, CA, USA, Sigma), using an avidin-biotin-peroxidase (ABC) (Hsu et al., 1981; Polak & Van Noorden, 1997) method. Sections were incubated with a specific commercial antibody raised in rabbits against rat ghrelin (Phoenix laboratories, CA, USA). The ghrelin antibody used for the immunocytochemical studies is stated to be specific (Phoenix Pharmaceuticals, Inc), since no cross-reactivity between ghrelin and any other peptide has been reported. The primary antibody was diluted 1:500 in 1-5% (v/v) normal goat serum (NGS), overnight, at 4 °C. After incubation, the slides were washed in PBS and incubated in ABC reagent for 1h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate diaminobenzidene tetrahydrochloride (DAB) (Sigma), which resulted in a brown colouration. The specificity of staining was determined by replacing the primary antibody with pre-immune rabbit serum or with PBS or by omission of the secondary antibody. As a positive control (Kojima et al., 1999) sections of the rat stomach were similarly stained for ghrelin immunoreactivity. Sections of the chick stomach and other areas of the gastrointestinal tract (crop, ileum, duodenum and colon) were similarly stained for ghrelin. Sections of the chicken stomach were also stained for somatostatin (SRIF) immunoreactivity, using an antibody raised in rabbits against SRIF₁₋₄₄ (Di Scala-Guenot et al., 1984) at a dilution of 1:200, since SRIF immunoreactivity has been found throughout the chicken gastrointestinal tract (Denbow, 2000).

In vivo GH-releasing activity

The GH-releasing activity of human (h) ghrelin (Phoenix Laboratories) was assessed in conscious 4 week old White Leghorn chicks, in comparison with h GHRH₁₋₄₄ (10 µg/kg) and TRH (10 µg/kg) (Bachem, Torrance, CA, USA). Leghorn chicken of 4-5 weeks of age were maintained indoors under natural lighting conditions with poultry food and tap water available *ad libitum*. Immature chickens (4-5 week old) were used in the

present study, since it is well established that the magnitude of the GH response is greater in young birds compared with older birds (Harvey & Scanes, 1984).

Groups of birds (n=8 to 16) were given a bolus i.v injection of peptide or the 0.9% w/v NaCl vehicle (1ml/kg body weight). Intravenous injections of ghrelin were administered through the right brachial vein, and heparinized blood samples were collected from the contralateral vein. During this procedure, the chickens were manually restrained and positioned on their sides. Ghrelin was administered at doses of 1, 3, 10 and 30 µg/kg, since this range includes the maximally effective dose of TRH and GHRH in chickens (Harvey & Scanes, 1984) and is comparable to doses of ghrelin that stimulate GH release in rats (4-20µg/kg in rats, Hosoda et al., 2000) and human (5µg/kg, Takaya et al., 2000; 33-66µg/kg, Seoane et al., 2000). Venous blood samples were collected from each bird 10 min after injection, at the time of the maximal GH response to TRH and GHRH (Harvey & Scanes, 1984).

Following centrifugation and separation, the plasma was stored at -20°C prior to GH radioimmunoassay (RIA), using NIDDK (National Institute of Diabetes and Digestive and Kidney diseases) reagents (Bethesda, Maryland, USA, kindly provided by Dr. A.L. Parlow).

In vitro GH-releasing activity

Chicken heads were obtained from a slaughter house (Lillydale, Edmonton, Canada) and were packed with crushed ice and transported to the laboratory for dissection. Pituitary glands were rapidly dissected from the heads and immediately placed into ice-cold Medium 199 (M 199) (Gibco laboratories, NY, USA) previously gassed with 95% O₂ / 5% CO₂. Pituitary glands (n=25 to 30) were diced into small pieces and incubated in 30 ml M 199 containing 20-40mg collagenase (Type VIII, Sigma) for 1-2 h in a shaking water-bath at 39°C. Cell dispersal was assisted by gently drawing the tissue in and out of a Pasteur pipette. The cells were then centrifuged at 1000g for 10 min, re-suspended in fresh M 199 and gently agitated. This washing procedure was repeated twice, and the cells re-suspended in a volume of M 199 to give a concentration equivalent to 1 pituitary/ml. The suspension was filtered through a piece of nylon gauze, and stirred

to ensure even distribution of cells. Aliquots (900 μ l) of the cell suspension were then incubated in the presence or absence of h ghrelin at final doses of 10^{-6} M – 10^{-8} M in 100 μ l M 199 in the treatment tubes (6 per treatment). The viability of the cells assessed by trypan-blue exclusion was >90% for each experiment. Different suspensions of pituitary cells were not corrected for differences in cell viability. Heterogeneous cell populations were used to reduce biological variability between individual pituitary glands. The treatment tubes were oxygenated 95% O₂ / 5% CO₂ and incubated for 2 hrs at 39°C in a shaking water-bath. Following incubation, the tubes were centrifuged at 2000g for 10 min, and 600 μ l supernatant was collected and stored at – 20°C prior to GH RIA.

Radioimmunoassay

Chicken (c) GH was measured using a double antibody RIA technique (Harvey & Scanes, 1977). Briefly, cGH (AFP7678B, NIDDK, USA) was iodinated by the chloramine T method. The reaction mixture contained 5 μ g hormone, 1mCi [¹²⁵ I] iodine (Amersham, Mississauga, ON, Canada), and 10 μ g chloramine T (Sigma, MO, USA) and was terminated after 20 sec by sodium metabisulphite. The tracer was then separated from damaged hormone and free Na-I¹²⁵ by gel filtration on a 24 x 1cm column of Sephadex G-100 (Pharmacia) (For details on the RIA technique see Appendix I).

GH was measured in a sample volume of 200 μ l and a final incubation volume of 400 μ l. Serial dilutions of a cGH standard (AFP9020C, NIDDK, USA) ranging from 1 – 500 μ g/l was constructed for each assay. Potency of the cGH standard from NIDDK are unknown. Each assay tube contained, tracer (¹²⁵ I-cGH; 10,000cpm), 1% NRS, RIA buffer, and primary antiserum (rabbit anti-cGH; final dilution 1:25,000; AFP55111186Rb, NIDDK, USA). After an overnight incubation, at 4°C, the secondary antibody (anti-goat IgG, whole molecule, Sigma) was added at a final dilution of 1:50. After an incubation for 48 h at 4°C , the tubes were centrifuged at 2700g at 4°C for 30 min and 5% (w/v) corn starch was added to cover the precipitate, After further

centrifugation (2700 g at 4°C for 10 min) the supernatants were discarded and pellet radioactivity was counted using a LKB gamma-master (Wallac OY, Turku, Finland).

Data analysis

GH release in response to human ghrelin, human GHRH or TRH stimulation was determined using a specific radioimmunoassay (RIA) for chicken (c) GH (Harvey & Scanes, 1977), and was expressed in terms of GH concentration (ug/l) in the plasma and media samples. Statistical differences in the results were determined by one-way analysis of variance (ANOVA) to indicate whether there were differences among the population means of the groups being compared (Bailer & Mosteller, 1992). Statistical differences between values obtained with various doses of each secretagogue were calculated by a Tukey post test (multiple-comparison technique) with a level of significance of P<0.05 (Graph Pad Prism, Version 2.1, 1996). To determine if the GH response was dose-dependent, linear regression (Bailer & Mosteller, 1992) was used to correlate the GH response with secretagogue dose (Graph Pad Prism, Version 2.1, 1996).

3. RESULTS

Immunocytochemistry

As expected (Kojima et al., 1999; Date et al., 2000a) ghrelin-ir was abundantly present within the X/A-like endocrine cells of the rat stomach (Fig. 2.1 A, C). Staining was completely abolished following the preabsorption of the antiserum with h ghrelin (Fig. 2.1 B). In contrast, sections of the chicken stomach was stained for somatostatin (SRIF) (Fig 2.2 A-C), since an absence of ghrelin immunoreactivity was observed with chicken stomach (Fig 2.3 A-D), crop, ileum, duodenum and colon (Fig 2.3 E & F, 2.4 A-F). In the hypothalamus, ghrelin-immunoreactivity was present in discrete cells in the nucleus anterior medialis hypothalami (AM) (Fig 2.5 A-C), and was also present in clusters of large ovoid cells in the nucleus magnocellularis preopticus pars medialis

(PRM) (Fig 2.5 A, E & F), nucleus magnocellularis preopticus supraopticus (PRS) (Fig 2.6 A-C) and in the chiasma opticus (CO) (Fig 2.6 A, E & F). Immunoreactivity for ghrelin was restricted to the cytoplasm of the perikarya and their axonal sprouts. Ghrelin staining was lost following the preabsorption of the antibody with h ghrelin (Fig 2.5 D & 2.6 D) and not seen when the ghrelin antibody was replaced by NRS or PBS (data not shown). Immunoreactivity for ghrelin was not present in any other hypothalamic nuclei (data not shown). The distribution of ghrelin immunoreactivity in the chicken hypothalamus is shown schematically in Fig 2.7.

In vivo induced GH release

In a preliminary study, circulating GH concentrations in conscious, immature chicks were promptly increased ($P<0.001$) following the i.v administration of h ghrelin (Fig 2.8 A). The increase in GH concentration (approximately 3 times that in controls) was comparable with that induced by the same dose (10 μ g/kg) of h GHRH, although less ($P<0.01$) than that (approximately 6 fold) induced by TRH. In a second study, circulating GH concentrations were also increased ($P<0.01$) following the administration of ghrelin at doses of 1, 3, 10 and 30 μ g/kg body weight (Fig 2.8 B). There was no difference between these groups in the plasma GH concentration.

In vitro induced GH release

Media content of GH from pituitary cells incubated with ghrelin were significantly increased ($P<0.05$), compared with controls (M 199 only) (Fig 2.9 A & B). The GH response to 10^{-6} M h ghrelin was significantly higher ($P<0.05$) than the response to 10^{-6} M h GHRH (Fig 2.9 A). GH release was increased ($P<0.001$) by each dose of ghrelin and the GH response to 10-6M ghrelin was greater ($P<0.05$) than the GH response to 10-8M ghrelin (Fig 2.9 B). Over this dose range, the GH response was dose-related ($r=0.96$ ($n=14$) $P<0.001$).

Figure 2.1. Ghrelin immunoreactivity in the rat stomach. (A): Ghrelin is present in the oxytic gland of the rat stomach x 100. (B): Ghrelin immunoreactivity is lost following preabsorption of the primary antibody with excess human (h) ghrelin x 100. (C): X/A like endocrine cells are strongly stained for ghrelin immunoreactivity x 1000.

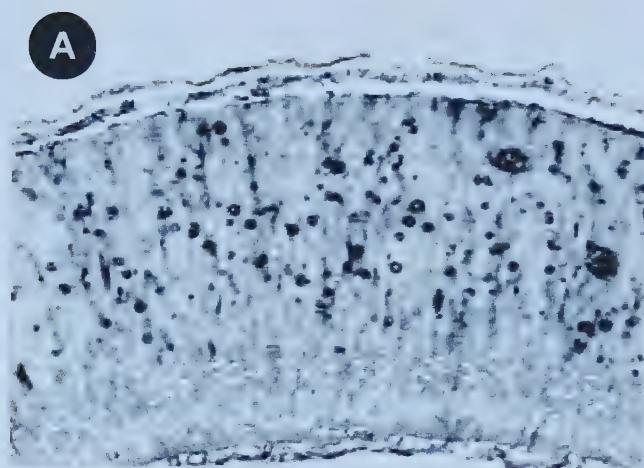


Figure 2.2. Somatostatin immunoreactivity in the chicken stomach.

(A): Somatostatin (SRIF) immunoreactivity in the chick stomach x 100. (B): SRIF immunoreactivity is lost following omission of the primary antibody x 100. (C): Highly stained D endocrine cells x 1000.



Figure 2.3. Absence of ghrelin immunoreactivity in the chicken stomach and crop.
No ghrelin immunoreactivity was found in any regions of the stomach or crop of the chicken, including the pylorus (top region of the stomach) (A) x 100 & (B) x 400, proventricularis (bottom region of the stomach) (C) x 100 & (D) x 400, and the crop (E) x 100 & (F) x 400.

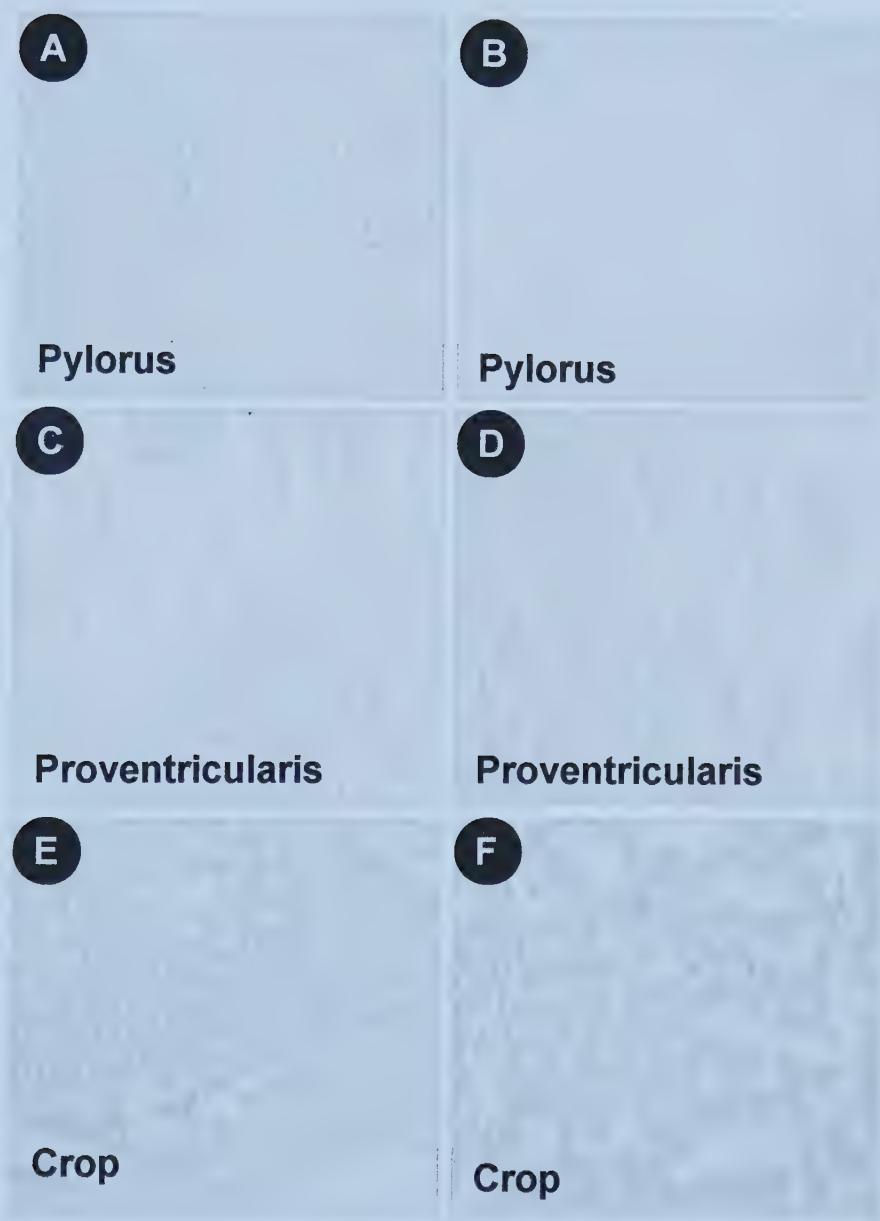


Figure 2.4. Absence of ghrelin immunoreactivity in the chicken intestine. No ghrelin immunoreactivity was found in any region of the intestinal tract of the chicken, including the ileum (A) x 100 & (B) x 400, the duodenum (C) x 100 & (D) x 400, and the colon (E) x 100 & (F) x 400.

A

B

Ileum

C

Ileum

D

Duodenum

E

Duodenum

F

Colon

Colon

Figure 2.5. Ghrelin immunoreactivity in the chicken hypothalamus I. (A): Ghrelin immunoreactive perikarya and fibres in the anterior medial hypothalamic nucleus (AM) and nucleus magnocellularis preopticus medialis (PRM) x 100. (B & C): Magnification of the AM x 400 & x 1000 respectively. (D): Ghrelin immunoreactivity is lost following preabsorption of the primary antibody with excess human (h) ghrelin x 100. (E & F): Magnification of the PRM x 400 and x 1000 respectively. Solid arrows indicate stained perikarya whilst dotted arrows indicate immunoreactive fibres. Abbreviations: LSO, organum septi laterale; VIII, third ventricle.

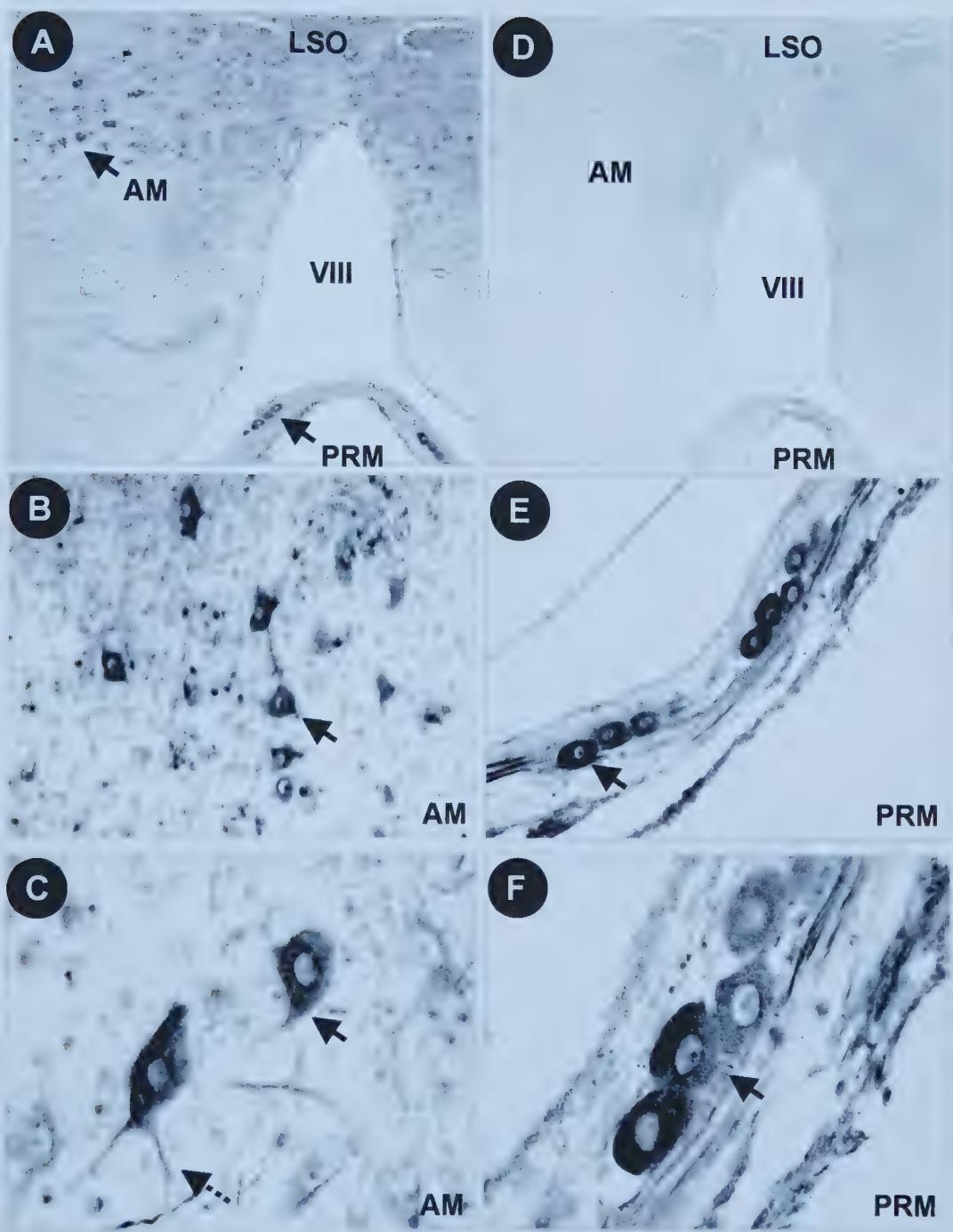


Figure 2.6. Ghrelin immunoreactivity in the chicken hypothalamus II. (A): Ghrelin immunoreactivity in perikarya and fibres in the chiasma opticus (CO) and nucleus magnocellularis preopticus pars supraopticus (PRS) x 100. (B & C): Magnification of the CO x 400 and x 1000 respectively. (D): Ghrelin immunoreactivity is lost following preabsorption of the primary antibody with excess human (h) ghrelin x 100. (E & F): Magnification of the PRS x 400 and x 1000 respectively. Solid arrows indicate stained perikarya whilst dotted arrows indicate immunoreactive fibres. Abbreviations: RP, recessus preopticus; VIII, third ventricle.

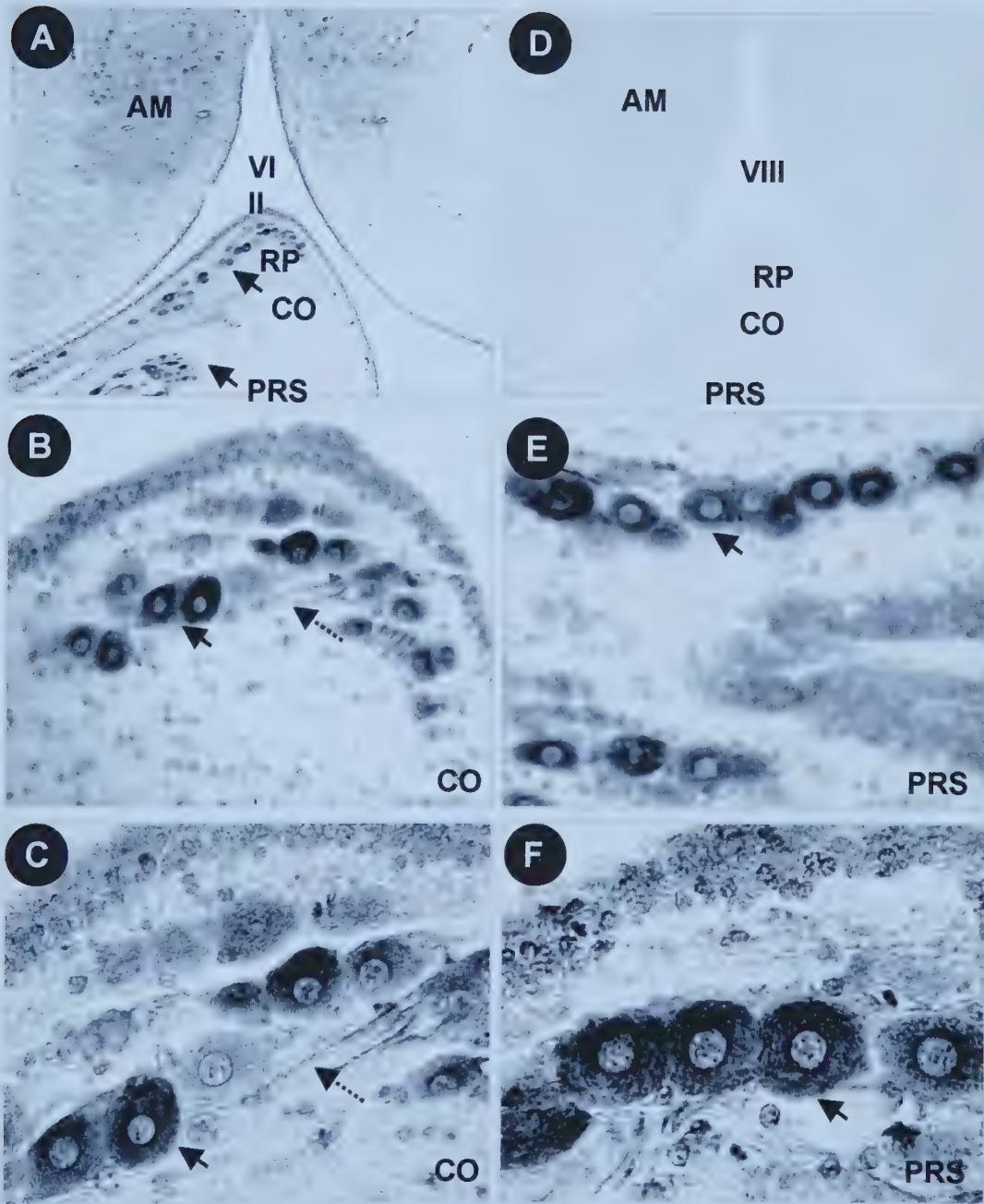


Figure 2.7. Schematic illustration of ghrelin immunoreactivity in the chicken hypothalamus. (A): Schematic illustration of the location of immunostained cell bodies (black and white circles) in the anterior hypothalamus (AM) and in magnocellular cells in the nucleus magnocellularis preopticus pars medialis (PRM). (B): Schematic illustration of the location of immunostained cell bodies (black and white circles) in magnocellular cells in the chiasma opticus (CO) and nucleus magnocellularis preopticus pars supraopticus (PRS). Abbreviations: SCN_M, nucleus suprachiasmaticus, pars medialis; FPL, fasciculus prosencephali lateralis (lateral forebrain bundle); LSO, organum septi laterale (lateral septal organ); RP, recessus preopticus; VIII, third ventricle). Based on the histological atlas of Kuenzel & Masson (1998).

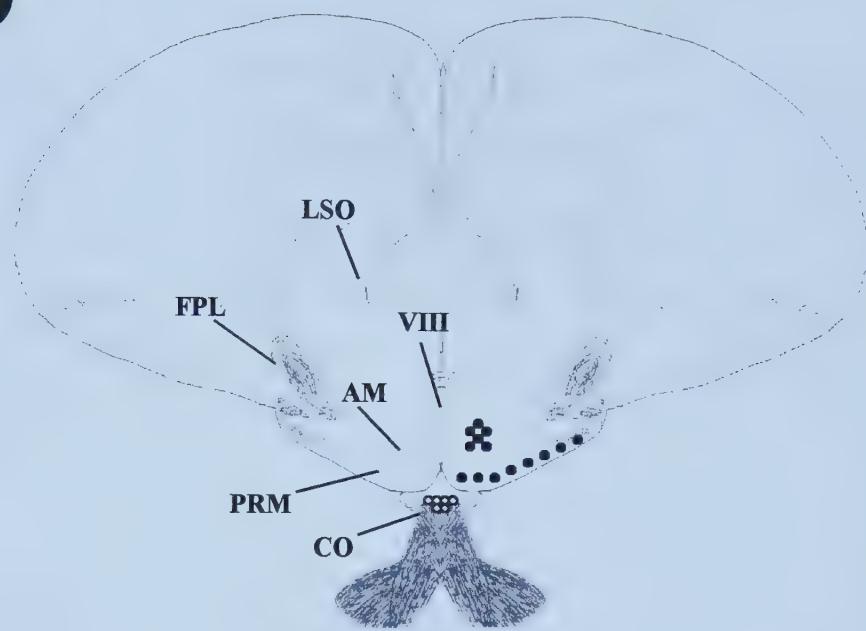
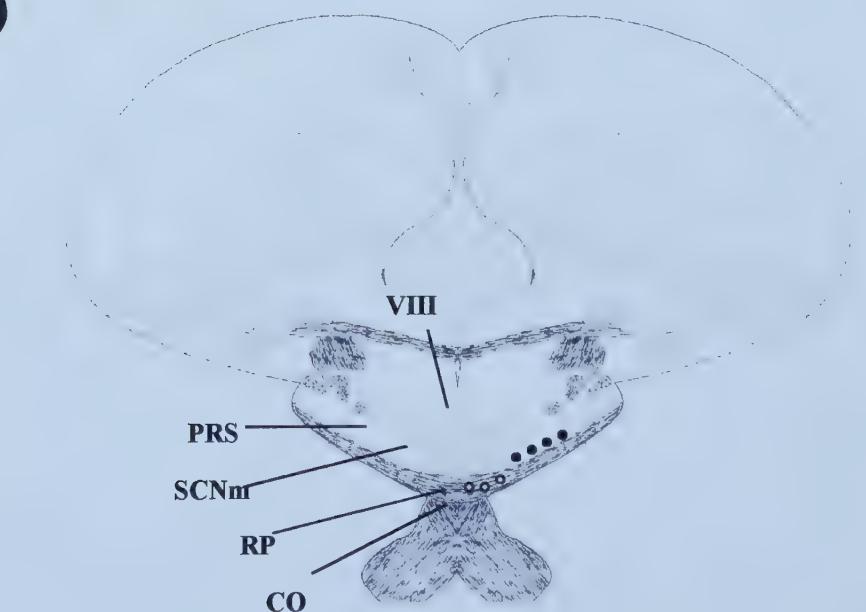
A**B**

Figure 2.8. The *in vivo* GH response to ghrelin in chickens. (A): Plasma growth hormone (GH) in 4-week-old chicks 10 min after the i.v injections of ghrelin, GHRH or TRH (all at 10 μ g/kg body weight), in comparison with controls injected (1 ml/kg body weight) with the 0.9% NaCl vehicle. (B): Plasma growth hormone (GH) in 4-week-old chicks 10 min after the i.v injections of ghrelin at 1, 3, 10 and 30 μ g/kg body weight. Groups significantly ($P<0.01$) different from the controls are indicated by asterisks. Numbers in each group are indicated in the bars. Means \pm SEM's.

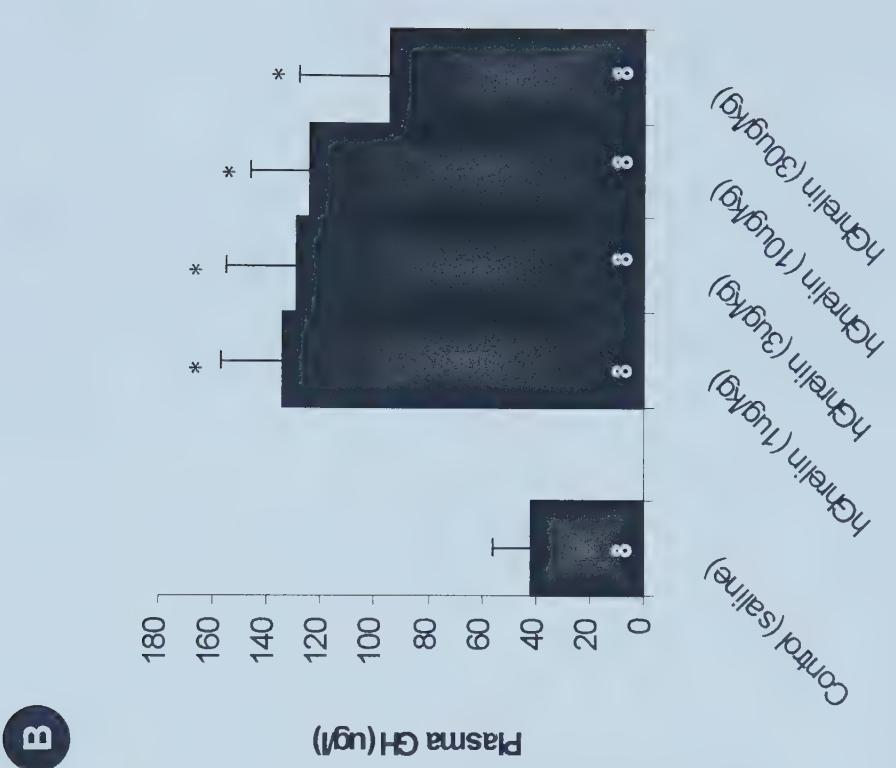
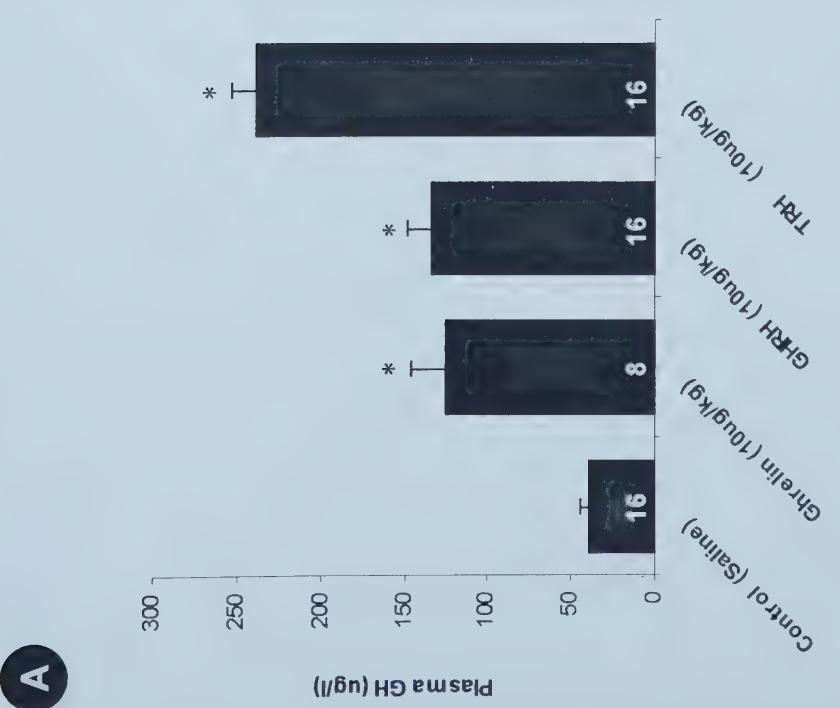
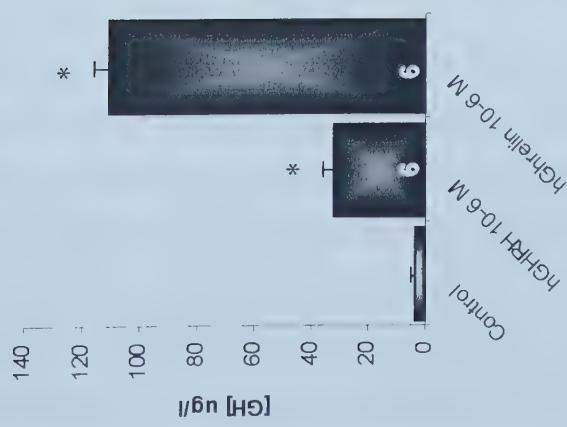
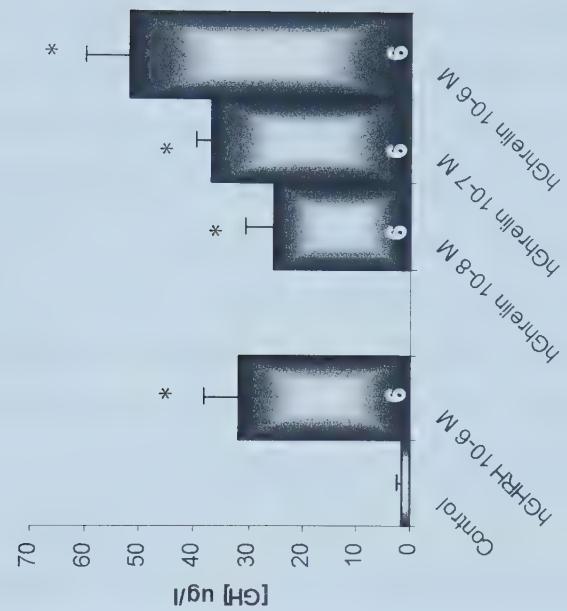


Figure 2.9. The *in vitro* GH response to ghrelin using chicken pituitary cells.

(A): Media content from chicken pituitary cells incubated with h GHRH and h ghrelin both at a dose of 10^{-6} M. (B): Media content from chicken pituitary cells incubated with ghrelin at doses ranging from 10^{-6} M – 10^{-9} M, in comparison with human (h) GHRH and controls (cells incubated with medium M199 only). Groups significantly ($P < 0.001$) different from controls are indicated by asterisks. Numbers in each group are indicated in the bars. Means \pm SEM's.

A

106

B

4. DISCUSSION

The results of the present study demonstrate, for the first time, the presence of a ghrelin-like peptide in a non-mammalian species. Ghrelin-immunoreactivity in the chick was, however, in dissimilar locations to its presence in mammals. In rats and humans, ghrelin is primarily found in the stomach and gastrointestinal tract (Kojima et al., 1999, 2001; Date et al., 2000a), although it is also present in the arcuate nucleus (Kojima et al., 1999; Korbonits et al., 2001a,b). In the chick, ghrelin immunoreactivity could not be detected in the stomach, duodenum, ileum or colon, even though somatostatin (SRIF) immunoreactivity was clearly present. This restriction of ghrelin immunoreactivity to the hypothalamus of the chick suggests it evolved phylogenetically as a neuropeptide rather than as a gastrointestinal hormone.

Although ghrelin was found in parvocellular cells in the hypothalamus, it was not found in the arcuate nucleus (infundibular nucleus) as in rats and humans, but in discrete populations in the anterior hypothalamus (AM). Since parvocellular cells in the hypothalamus are thought to terminate on portal vessels in the median eminence, the presence of ghrelin in the AM suggests it may be a hypothalamo-hypophysial releasing factor and stimulate pituitary GH release after secretion into the hypothalamo-hypophysial circulation. Furthermore, as GH has recently been discovered in the AM (Ramesh et al., 2000), ghrelin may also have roles in the autocrine or paracrine regulation of GH synthesis or release within the brain.

In addition to the AM, ghrelin immunoreactivity was also found in magnocellular cells in the ventromedial hypothalamus [in the PRM and PRS (derived from the supraoptic nucleus) and in the CO]. This is the first demonstration of ghrelin in magnocellular cells. The presence of ghrelin in these cells therefore resembles the presence of arginine vasotocin and Mesotocin in the chicken hypothalamus (Tennyson et al., 1985; Robinzon et al., 1988; Barth et al., 1997). Fibres from these cells have been shown to extend to the avian median eminence (Mikami, 1985; Tennyson et al., 1985; Robinzon et al., 1988) and it is, therefore, possible that ghrelin stimulates pituitary GH after release from these fibres into the hypophysial portal circulation. Most magnocellular

neurons do not, however, terminate in the median eminence but in the neurohypophysis. Ghrelin-induced GH secretion in the chick may thus be indirect and mediated by release into the short portal vessels connecting the pars nervosa with the pars distalis. Although indirect, this route is commonly utilized for transporting hypothalamic-releasing factors to the adenohypophysis in non-primate species (Anthony et al., 1998).

These results also demonstrate, for the first time, a stimulatory effect of human (h) ghrelin on GH secretion in the chicken *in vivo*. The prompt GH-releasing activity of h ghrelin was comparable to that induced by the same dose (10 μ g/kg) of h GHRH, although of lesser magnitude than that induced by TRH, on a weight basis. It is, therefore, of interest that activation of the GHS-R (ghrelin-R) by two non-peptidyl mimics (L-692, 429 and L-163, 255) also promptly increased GH secretion in the chicken (Geris et al., 1998, 2001). Ghrelin-induced GH release *in vivo* was not dose-dependent over the range of doses used. However, this could simply reflect a narrow dose range over which threshold levels were met and saturation of receptors may have occurred. The slight decrease in GH response to 30 μ g/kg ghrelin *in vivo* may represent an inhibitory feedback mechanism, which results in desensitization of somatotrophs and possibly downregulation of the GHS-R. Therefore, additional dose- and time-course studies are required to fully characterize its role as a GH-secretagogue (GHS) in birds, especially as these preliminary studies indicate that doses of ghrelin between 1-30 μ g/kg are equally effective. The present study also demonstrated a direct pituitary action of ghrelin in dispersed chicken pituitary cells *in vitro*. This is consistent with the presence of a GHS (ghrelin)-R in the chick pituitary gland (Toogood et al., 1998; Gaylinn et al., 2000), the stimulatory actions of peptidomimetics on the release of GH from perfused chicken pituitary glands (Geris et al., 1998, 2001) and the ability of ghrelin to induce GH release from rat pituitary glands *in vitro* (Kojima et al., 1999). The GH response of chicken pituitary cells to ghrelin *in vitro* was dose-dependent, in contrast with the GH response *in vivo*. The large variation between the two trials in the magnitude of the ghrelin-induced GH response *in vitro* can also be explained by: 1) differences in cell viability (trypan-blue exclusion tests were performed to establish that a reasonable percentage of the cell populations were viable, however, cell number was not corrected for differences in

viability); 2) differences in incubation periods with collagenase for cell dispersal (excessive use of collagenase may have caused cell or receptor damage); 3) differences in strains of chickens obtained from the slaughter house (differing strains may exhibit variations in GH response).

In addition to a pituitary site of action, ghrelin-induced GH secretion may, however, also have been mediated centrally. Indeed, ghrelin has been shown to rapidly (within 10 min) stimulate GH secretion in rats through an inhibition of hypothalamic somatostatin release (Date et al., 2000b; Tolle et al., 2000; Wren et al., 2000). Interestingly, SRIF-ir perikarya have been found to overlap with hypothalamic magnocellular nuclei containing AVT and MT in birds (Mikami & Yamada, 1984), therefore it is not surprising that ghrelin was also found in these magnocellular nuclei, as ghrelin could act as a functional antagonist of SRIF to indirectly promote GH release by locally suppressing SRIF release. This can be supported by reports that suggest that GHSs antagonize SRIF at the hypothalamic level to diminish SRIF tone on GHRH-containing neurons in mammals (Conley et al., 1995; Tolle et al., 2000).

Hypothalamic actions of ghrelin on fos-, erg-1, NPY, and agouti-related protein transcription (Hewson & Dickson, 2000; Nakazato et al., 2001) and food intake (Kamegai et al., 2000; Tschop et al., 2000; Masuda et al., 2000; Nakazato et al., 2001; Shintani et al., 2001) are also well established, reflecting the widespread distribution of ghrelin receptors in the CNS (Howard et al., 1996; McKee et al., 1997; Guan et al., 1997). The GH-releasing activity of h ghrelin in the chicken may, thus, be partially mediated by actions at CNS sites, especially as nonpeptidyl mimetics of GHS receptors partially stimulate GH release in chicks by rapidly increasing hypothalamic TRH release (Geris et al., 2001). The stimulatory effects of GHRH and TRH on GH secretion in the chick are similarly partially mediated by actions within the CNS (Harvey, 1990, 1999). The possibility that ghrelin acts within the CNS is also supported by its ability to act at central sites to modulate food intake in the fowl (Furuse et al., 2001).

In summary, these results demonstrate the presence of ghrelin immunoreactivity in discrete populations of the AM and magnocellular neurons of the chick hypothalamus and stimulatory actions of ghrelin on GH secretion in the chick.

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CHAPTER THREE

Growth hormone and ghrelin in neural tissues of early chick embryos.



Some of the results in this chapter were presented at the 9th Annual Western Perinatal Research Conference. Ahmed, S & Harvey, S (2001) Extrapituitary growth hormone (GH) in chick embryos: Comparable distribution of ghrelin, a GH-secretagogue. Banff, Canada (Abstract 19). Data from this chapter was also presented at the 34th International Congress of Physiological Sciences. Ahmed, S. & Harvey, S (2001) Extrapituitary growth hormone (GH) in chick embryos: Comparable distribution of ghrelin, a GH-secretagogue. Christchurch, New Zealand (Abstract 1455).

1. INTRODUCTION

Ghrelin, a growth (GH)-releasing factor (GRF) is present in the hypothalamus of rats (Kojima et al., 1999), humans (Korbonits et al., 2000) and chickens (Ahmed & Harvey, 2001). It is, however, also present in the stomach of rats, sheep, goat, cows and humans (Kojima et al., 1999; Date et al., 2000a,b; Gualillo et al., 2001; Hayashida et al., 2001), and in the mammalian gastrointestinal tract (in the duodenum, ileum, colon and jejunum) (Date et al., 2000a,b; Hosoda et al., 2000), the mouse kidney (Mori et al., 2000) and in the rat and human placenta (Gualillo et al., 2001). This wide distribution is similar to the almost ubiquitous distribution of GH-releasing hormone (GHRH) (Bosman et al., 1984; Weigent et al., 1991; Bagnato et al., 1992; Petersenn et al., 1998) and somatostatin (Stuesse et al., 2001; Alexander et al., 2001; Roudenok & Kuhnel, 2001; Xiang et al., 2001), which also have physiological roles as hypophysiotropic factors in GH regulation. These GRFs may thus have numerous, perhaps tissue-specific roles unrelated to GH secretion (Campbell & Scanes- review of GHRH; Janecka et al., 2001- review of SRIF; Inui, 2001; Kojima et al., 2001- reviews of ghrelin). The widespread distribution of these GRFs may, however, also reflect a widespread expression of the GH gene.

It is now well established that GH gene expression is not confined to pituitary somatotrophs and occurs in many extrapituitary sites, in which the co-localization of GHRH and SRIF may represent a local “hypothalamo-pituitary” axis (Harvey & Hull, 1997). It is, therefore, of interest that ghrelin is present in tissues in which GH has also been found in the hypothalamus, the stomach- in the gastrointestinal tract in the kidney, in the placenta (Kyle et al., 1981; Castro et al., 1993). The presence of ghrelin in sites of GH synthesis is further demonstrated by ghrelin gene expression in the pituitary gland (Korbonits et al., 2001) and by its induction of pit-1 (Garcia et al., 2001), a transcription factor involved in somatotroph differentiation and GH secretion in pituitary and extrapituitary sites (Harvey et al., 2000).

The possible presence of ghrelin in early chick embryos has therefore been determined in the present study, since GH and pit-1 are present in neural tissues of chick

embryos prior to the differentiation of the pituitary gland (Harvey et al., 2000, 2001; Murphy & Harvey, 2001a,b).

2. MATERIALS & METHODS

Tissues

Fertile White Leghorn eggs (University of Alberta, Poultry Research Centre) were incubated at 37°C in humidified air (Hamburger & Hamilton, 1951). The eggs were turned one quarter of a revolution each day during incubation. At embryonic day (ED) 7, embryo heads were collected in ice-cold phosphate buffered saline (PBS; pH 7.4). ED 7 embryos were selected for the study, because ED 7 occurs at the end of organogenesis but before the differentiation of the pituitary gland, and, because GH and pit-1 are present in neural tissues of the developing brain (Murphy & Harvey, 2001a,b). Neural structures in the head were identified by the embryological atlas of Bellairs & Osmond (1998).

Immunocytochemistry

Embryo heads were fixed in freshly prepared paraformaldehyde (4% w/v) (Sigma, Mississauga, Ontario, Canada) and left to fix overnight at 4°C. Tissues were then dehydrated in a graded series of alcohol (50% v/v, 15-30 min; 70%, 30-60 min; 95%, 30-60 min; 100%, 60-120 min) and cleared with hemo-de (a de-paraffinizing agent) (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax for 24 h at 60 °C, under normal atmospheric pressure. Serial transverse sections (4-8µm) were cut with a microtome and mounted onto treated slides (Fisher Scientific).

Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlington, CA, USA, Sigma), using an avidin-biotin-peroxidase (ABC) (Hsu et al., 1981; Polak & Van Noorden, 1997) method. Sections were incubated with a

specific antibody, raised in rabbits against chicken (c) GH (Harvey & Scanes, 1977) or with a specific commercial antibody raised in rabbits against rat (r) ghrelin (Phoenix laboratories, CA, USA). CA, USA). The ghrelin antibody used for the immunocytochemical studies is stated to be specific (Phoenix Pharmaceuticals, Inc), since no cross-reactivity between ghrelin and any other peptide has been reported. The GH antibody was diluted 1: 1000 and the ghrelin antibody was diluted 1:500 in 1-5% normal goat serum (NGS), overnight, at 4 °C. After incubation, the slides were washed in PBS and incubated in ABC reagent for 1h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate diaminobenzidene tetrahydrochloride (DAB) (Sigma), which resulted in a brown colouration. The specificity of staining was determined by replacing the primary antibody with pre-immune rabbit serum or by preabsorption of the primary antibodies with excess (100ug/ml) c GH or r ghrelin, respectively.

The GH antibody has been previously used to detect GH immunoreactivity in neural tissues of chick embryos (Harvey et al., 2001; Murphy & Harvey, 2001), and its specificity is indicated by its labeling of somatotrophs in the chicken pituitary gland (Fig 3.1 a & b). The ghrelin antibody has also been used to label specific immunoreactive cells in the hypothalamus of neonatal chicks (Ahmed & Harvey, 2001).

3. RESULTS

As expected (Murphy & Harvey, 2001), GH immunoreactivity was present in the ED 7 brain. It was particularly intense in the walls of the mesencephalon, (Fig 3.2 A), infundibulum (Fig 3.2 C), and diencephalon (Fig 3.2 E & G). It was also present in the right accessory nerve (Fig 3.2 A) and the ependymal cells of the choroid plexus (Fig 3.2 E). Ghrelin immunoreactivity was similarly present and abundant in the same tissues (Fig 3.2 B, D, F & H). GH- and ghrelin-immunoreactivity was also localized in cells lining the otic vesicle (Fig 3.3 A & B) and in the trigeminal nerve (Fig 3.3 C & D). The specificity of the GH- and ghrelin- staining was indicated by its loss when the primary antibodies

were replaced by pre-immune rabbit serum (data not shown) or when the GH antibody was preabsorbed by excess GH (data not shown). The specificity of the ghrelin antibody is shown by the loss of staining, following its preabsorption, in cells surrounding the diocoel (Fig 3.4 A), diencephalon (Fig 3.4 B), choroid plexus (Fig 3.4 B), infundibulum (Fig 3.4 C), mesencephalon (Fig 3.4 D) and otic vesicle (Fig 3.4 E).

In addition to neural tissues in the ED 7 head, ghrelin immunoreactivity was also clearly present in surrounding mesodermal and ectodermal cells (Fig 3.5 C & D). In marked contrast, GH immunoreactivity was not present in these tissues (Fig 3.5 A & B). The ghrelin staining in the ectodermal and mesodermal cells was completely lost following the preabsorption of the antibody with excess r ghrelin (Fig 3.5 E & F).

Figure 3.1. Growth hormone (GH) immunoreactivity in the adult chicken anterior pituitary gland. (A): This transverse section illustrates the distribution of immunoreactive somatotrophs in the caudal lobe (Ca) using an antibody against native chicken GH. Isolated cells and small groups (clumps) are also found in the cephalic lobe (Ce) of the pituitary gland x 40. (B): Magnification of somatotrophs of the caudal lobe x 100.

A



Ca

Ce

B

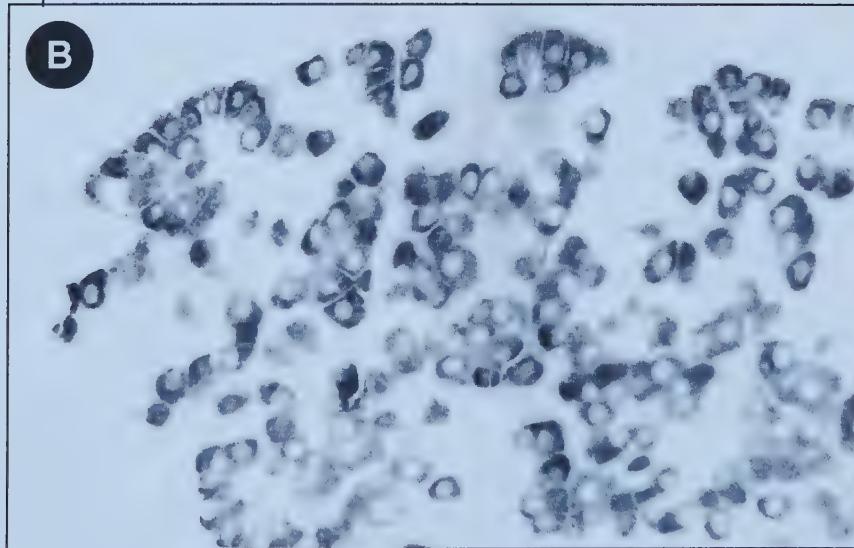


Figure 3.2. Growth hormone (GH) and ghrelin immunoreactivity in neural tissues of ED 7 chick embryo. (A & B): Intense GH and ghrelin staining in the mesencephalon (m) of the developing brain respectively x 400. Abbreviations: right accessory nerve (ran); otic vesicle (ov). (C & D): GH and ghrelin staining found in the infundibulum x 400. (E & F): Strong GH and ghrelin staining in the diencephalon (d) and in the choroid plexus (cp) x 400. (G & H): Immunoreactive GH and ghrelin in the ependymal cells surrounding the diocoele respectively x 400.

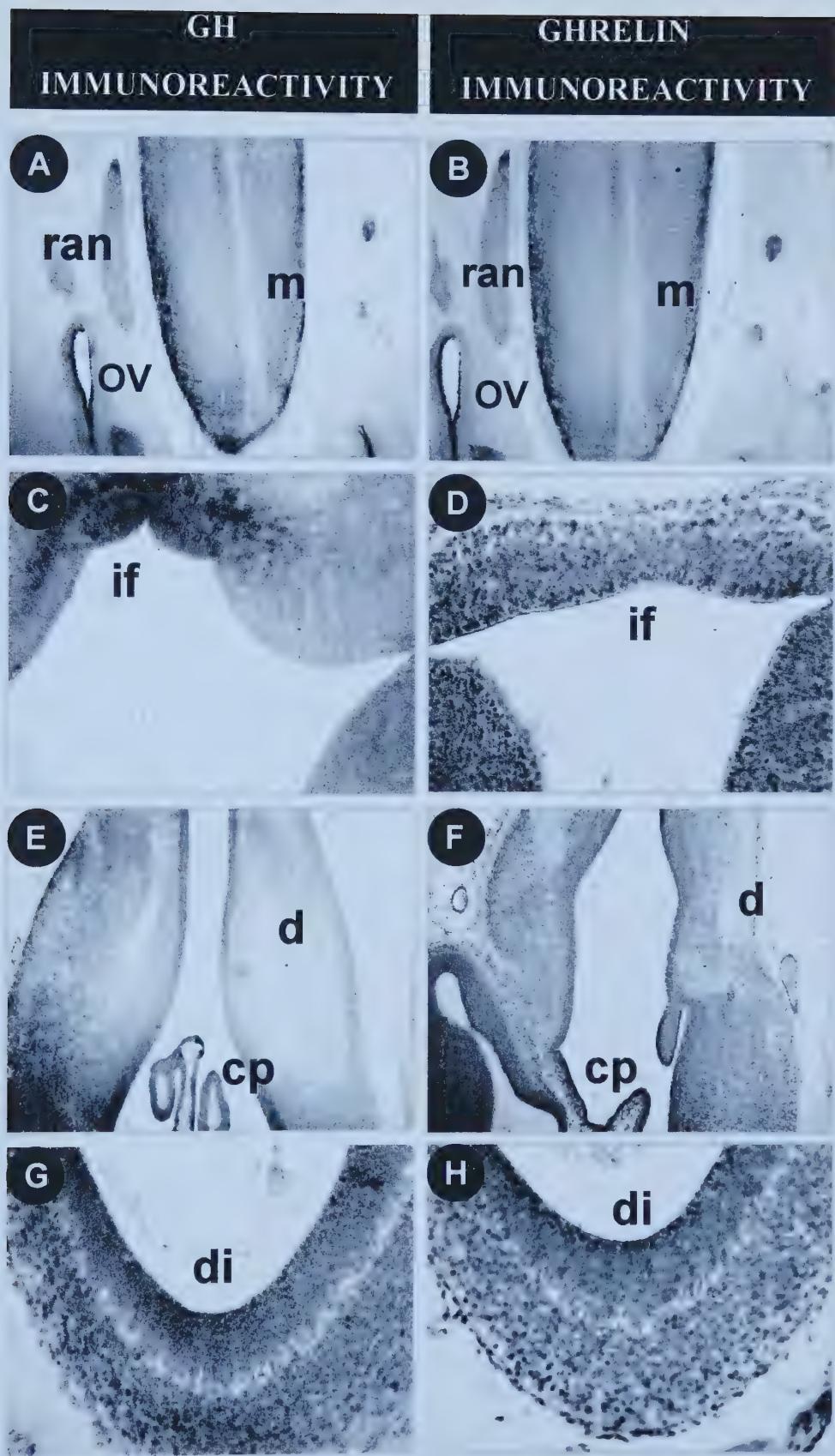


Figure 3.3. Growth hormone (GH) and ghrelin immunoreactivity in the otic vesicle and trigeminal ganglion of the ED 7 chick embryo head. (A & B): Immunoreactive GH and ghrelin in the endothelial cells lining the otic vesicle respectively x 400. (C & D): Strong staining for GH and ghrelin was also present in the trigeminal ganglion of the ED 7 chick embryo head respectively x 400.

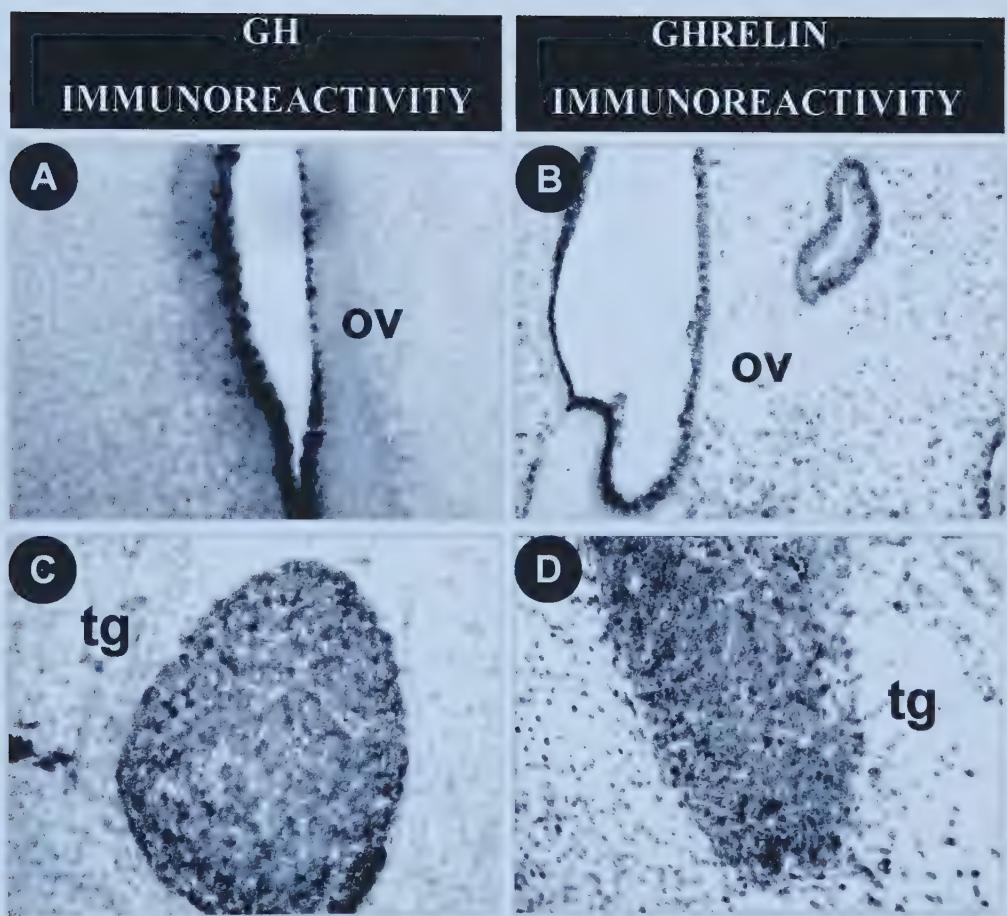


Figure 3.4. Negative controls/preabsorption controls for ghrelin staining in the ED 7 chick embryo. (A-F): Ghrelin immunoreactivity is lost following preabsorption of the anti-rat ghrelin antibody with excess human (h) ghrelin respectively x 100. Preabsorption of the anti-chicken (c) GH antibody with excess recombinant cGH also resulted in a loss of immunoreactivity (data not shown). Negative controls for ghrelin staining in (A): diocoel (di) x100; (B): diencephalon (d) and choroid plexus (cp) x 400; (C): infundibulum x400; (D): mesencephalon (m) x400; (E): otic vesicle (ov) x400; (F): ectodermal (ec) and mesodermal (ms) layers of the embryo head x 400.

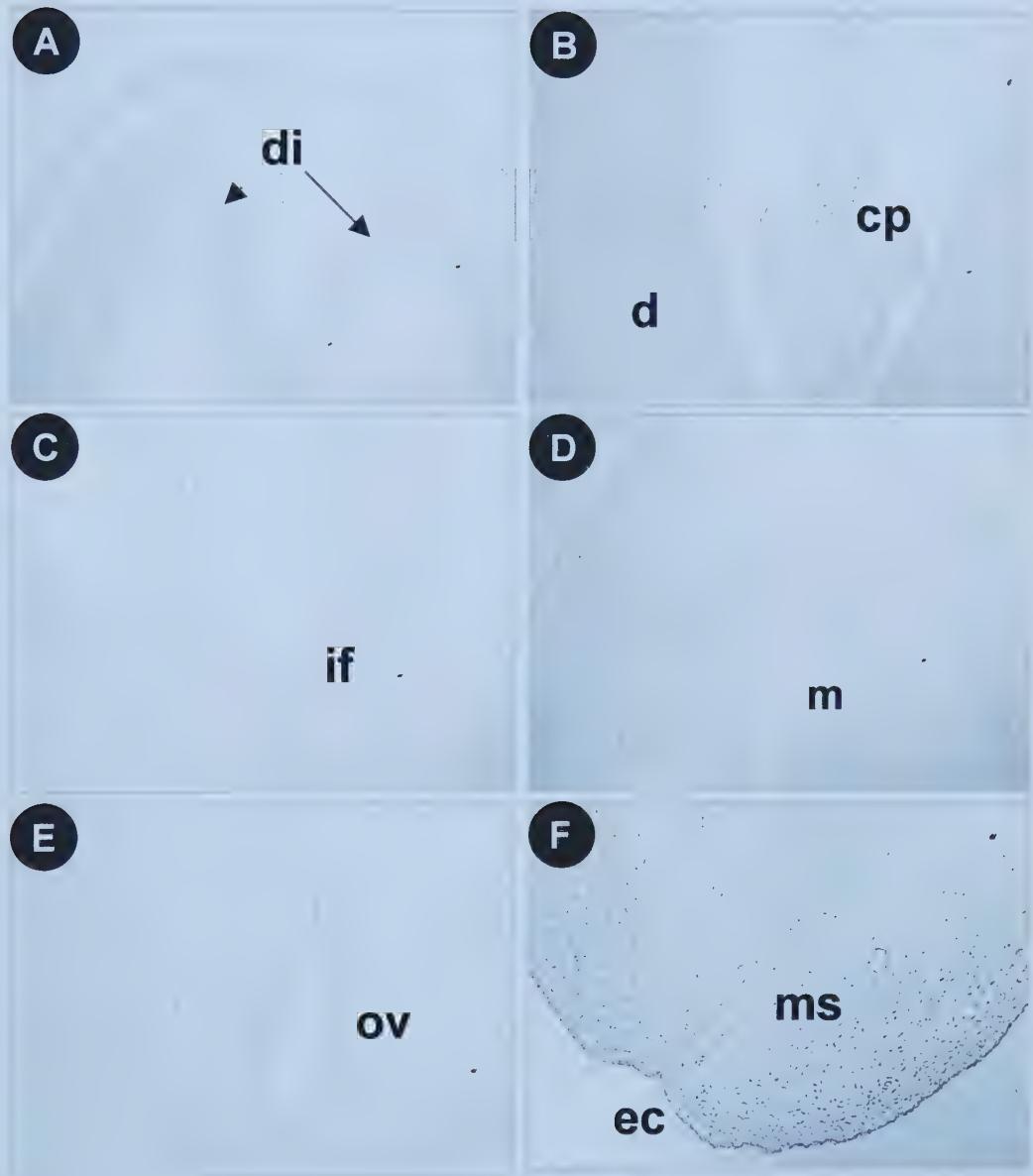


Figure 3.5. Ghrelin immunoreactivity in ectodermal and mesodermal layers of the ED 7 chick embryo head. Ghrelin-immunoreactivity was particularly strong in ectoderm and mesoderm layers in posterior and anterior regions of the head, in which GH-immunoreactivity was absent. (C & D): Ghrelin immunoreactivity in the ectoderm and mesoderm layers of the embryo head x 400. (A & B): An absence of GH immunoreactivity in the ectoderm and mesoderm layers of the embryo head x 400. (E & F): Preabsorption controls for the ghrelin staining x 400.

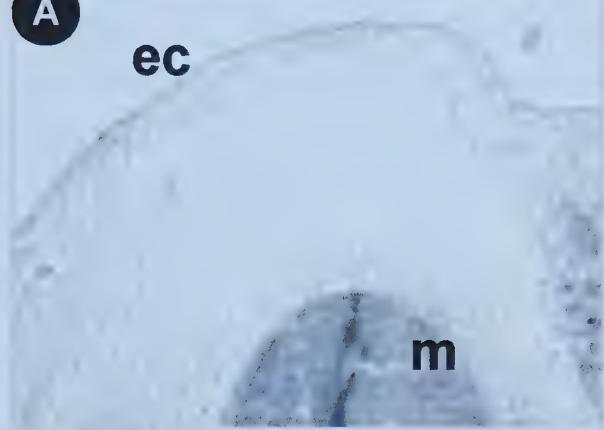
GH

IMMUNOREACTIVITY

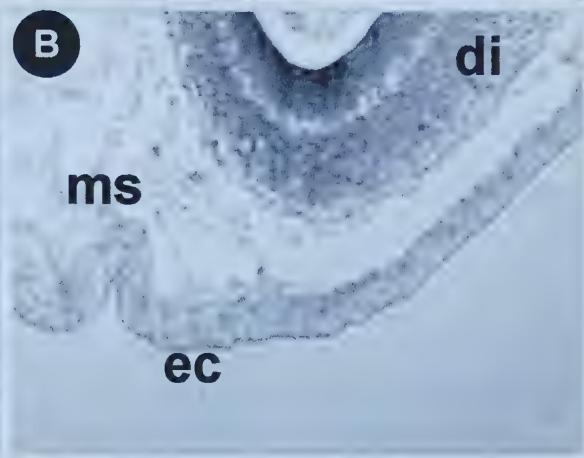
GHRELIN

IMMUNOREACTIVITY

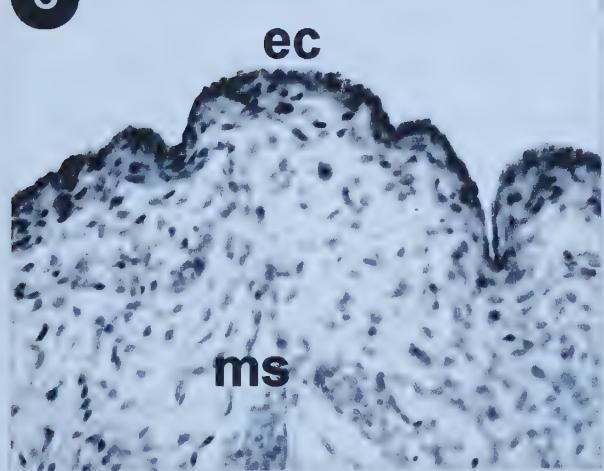
A



B



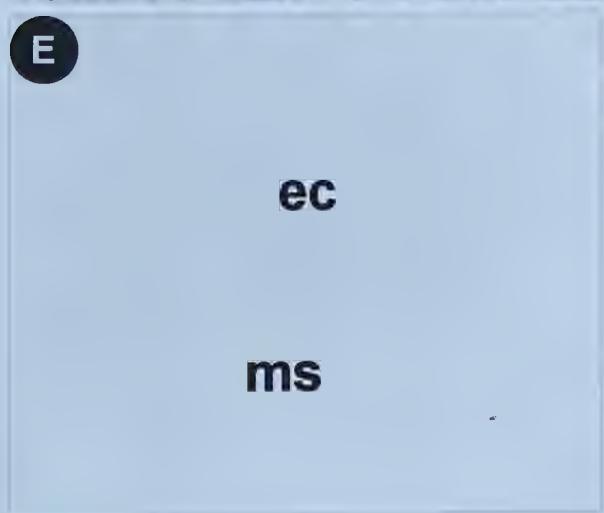
C



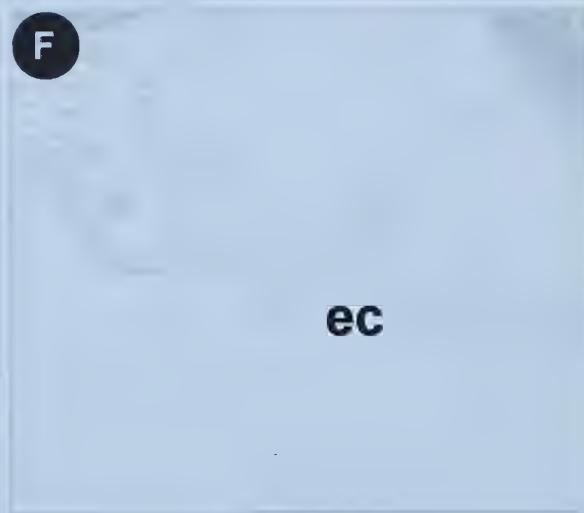
D



E



F



4. DISCUSSION

These results demonstrate that ghrelin immunoreactivity is present and widely distributed in neural tissues of early chick embryos, with a distribution similar to that of GH immunoreactivity. It is, therefore, possible that ghrelin regulates GH release in neural tissues of the embryo, as ghrelin stimulates pituitary GH release in neonatal chicks (Ahmed & Harvey, 2001). Moreover, as ghrelin induces pit-1 (pituitary-specific transcription factor) expression in the rat pituitary (Garcia et al., 2001) and as pit-1 stimulates GH synthesis in pituitary and extrapituitary sites (Harvey et al., 2000), ghrelin may stimulate GH synthesis in the embryonic chick brain. The presence of pit-1 in the same neural tissues of the chick embryo (Murphy & Harvey, 2001a) strongly supports this possibility. The presence of GH receptors in neural tissues of ED 7 embryos (Harvey et al., 2001) therefore indicates that an elaborate autocrine/paracrine system of GH synthesis and action is present in the brain during embryogenesis. It would therefore be of interest to determine if ghrelin is similarly present in other extrapituitary sites of GH synthesis.

Although ghrelin immunoreactivity is widespread in neural tissues of the chick embryo, it is confined to a small number of discrete magnocellular cells in the hypothalamus of neonatal chicks (Ahmed & Harvey, 2001), and to a small number of parvocellular cells in the hypothalamus of rats (Kojima et al., 1999) and humans (Korbonits et al., 2001). This suggests an ontogenetic extinction of ghrelin expression, and it is therefore of interest that GH expression in the brain is also restricted to hypothalamic nuclei after birth in rats (Hojavat et al., 1982) and to a relatively small number of hypothalamic and extrahypothalamic nuclei after hatch in turkey and dove brains (Ramesh et al., 2000). It is, therefore, possible that neural GH expression is ghrelin-dependent perinatally and neonatally. Although the present study demonstrated a similar distribution of ghrelin and GH immunoreactivity in extrapituitary sites of the ED 7 chick embryo, the possibility of GH and ghrelin co-localization in the same cells was not addressed. Co-localization studies are, therefore, needed to establish if ghrelin has paracrine actions on GH release. Such studies could involve the use of double staining

techniques, which use two different coloured chromogens. If ghrelin and GH are indeed co-localized, the resulting staining will be the combination colour of the two chromogens applied.

The presence of ghrelin in the hypothalami of neonatal chicks (Ahmed & Harvey, 2001) was thought to indicate its phylogenetic evolution as a neuropeptide, since it was not present in the chicken gastrointestinal tract. The widespread presence of ghrelin in neural tissues of the chick embryo supports this view. This finding contrasts, however, with the presence of ghrelin in the stomach of fetal sheep and the absence of ghrelin immunoreactivity in fetal sheep hypothalami (Roelfsema et al., 2001). Ghrelin may thus have a different ontogeny or have different roles in birds and mammals. It would, therefore, be of interest to investigate if ghrelin immunoreactivity is present in regions other than the tissues of the ED7 chick embryo head. For instance, sections throughout the ED7 chick embryo body should be similarly stained for ghrelin, in particular the gastrointestinal loop of the embryo, as demonstrated in fetal sheep (Roelfsema et al., 2001). If ghrelin immunoreactivity is detected in the chick embryo gut, this will demonstrate a differential regulatory pattern of ghrelin expression in terms of ontogenetic extinction, since ghrelin is absent in the gastrointestinal tract of chickens (Ahmed & Harvey, 2001). GH is also detected in the chick embryo body (Harvey et al., 2000), therefore, the presence of ghrelin in similar body regions would indicate the possibility of GH actions also being ghrelin-dependent in these tissues in embryogenesis.

In addition to GH regulation, the widespread presence of ghrelin (GHS) receptors in mammals (Papotti et al., 2000) suggests it has numerous roles other than GH regulation. The finding of ghrelin immunoreactivity in ectodermal and mesodermal cells of the ED 7 chick embryo head that had no GH immunoreactivity also suggests GH-independent roles for ghrelin in birds. These roles are, however, uncertain.

In summary, ghrelin staining was intense and widespread and in some GH immunoreactive tissues, indicating that extrapituitary GH production may be ghrelin-dependent during early embryogenesis. A functional autocrine/paracrine cascade resulting in GH production and action thus appears to be present in early chick embryos.

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CHAPTER FOUR

General Discussion



The recent discovery of ghrelin by Kojima et al., (in December 1999) was a landmark in neuroendocrine research. It has provided insight into the roles of GH in feeding and metabolism and led to a revision of the doctrinaire models of GH secretion.

Ghrelin, in mammals, is primarily a 'gut' peptide found in the stomach and the gastrointestinal tract. It is thought to be released into the systemic circulation and to act at the pituitary gland, to stimulate GH release directly, or indirectly after crossing the blood-brain-barrier and inducing GHRH release (Dickson, 1999; Tannenbaum & Bowers, 2001). It also acts centrally to induce appetite (ghrelin is the most powerful stimulator of appetite of all known orexigenic peptides). Ghrelin, in mammals, is thus thought to provoke GH release, for the stimulation of body growth and protein anabolism (synthesis of organic molecules required for cell structure and function), as well as, to increase feeding, to provide an overall positive energy balance. The increase in GH by ghrelin would thus serve to increase gluconeogenesis (provides glucose during fasting) by the liver, induce adipocyte (fat cells) lipolysis (fat breakdown) and inhibit insulin's ability to cause glucose uptake by peripheral tissues. The increase in food intake by ghrelin would, therefore, directly increase nutrient levels in plasma. It is also important to note that ghrelin's GH-releasing activity and appetite-stimulating ability can occur independently of each other, since ghrelin increases body weight gain as a result of overeating in GH-deficient dwarf rats (Tschop et al., 2000).

In mammals, GH increases food intake during fasting whilst GH levels decrease in the post-absorptive (fed) period. During food restriction (starvation) GH levels will rapidly increase to promote feeding in most animals (Sonntag et al., 1995). Therefore an increase in GH release by either ghrelin or GHRH stimulation could also result in feeding (Vaccarino et al., 1985; Wren et al., 2000). It is, however, interesting to note that no studies thus far have simultaneously investigated the combined effects of ghrelin on GH secretion and appetite control in any species. However, it has been postulated that, in mammals, ghrelin is involved in regulating energy balance (Horvath et al., 2001). Ghrelin can either increase energy levels directly (via an increase in nutrient intake) or indirectly

(via an increase in GH secretion, which also increases feeding). However, no reports have demonstrated a significant correlation between plasma ghrelin levels and circulating GH or IGF-1 levels. Furthermore, the orexigenic effects of ghrelin can occur independently of its GH-releasing activity in rats (Tschoop et al., 2000). It has also been suggested that ghrelin's actions on GH regulation and feeding may be mediated by different ghrelin/GHS-R subtypes (Torsello et al., 2000; Toth et al., 2001). Therefore, detailed investigations of GHS-R subtypes and the dual action of ghrelin on GH and food regulation are warranted.

A role for ghrelin in feeding behaviour in mammals is supported by its abundance in the stomach (an organ that senses fluxes in energy balance), its rapid (within 20 min) ability to increase food intake and gastric acid secretion in rats in both light (satiated) and dark (feeding) phases, and its co-localization in and activation of NPY/AGRP (orexigenic peptides) neurons (Cummings et al., 2001). Ghrelin may not, however, be an orexigenic peptide in chicks, in which it inhibited food intake (Furuse et al., 2000). The absence of ghrelin in the gastrointestinal tract of chickens (Ahmed & Harvey, 2001) and its absence in the avian ARC nucleus (site of orexigenic peptide production) are consistent with this view. It is also pertinent that motilin similarly stimulates feeding in mammals but suppresses feeding in chickens (Ando et al., 2000). Furthermore, since ghrelin is not found in the avian ARC nucleus but instead is present in magnocellular nuclei within the hypothalamus, it can be speculated that ghrelin is anorexigenic itself or induces the release of other anorexigenic factors found in close association with the magnocellular neurons containing ghrelin. The significance of such species-dependent actions of ghrelin on food regulation is not currently understood.

In addition to the gut, ghrelin is also present in the mammalian brain. The presence of neuropeptides in hypothalamic neurons that have axon terminals on portal blood vessels in the median eminence suggests they are involved in pituitary regulation, after release into pituitary portal circulation. The presence of ghrelin in the arcuate nucleus of mammals, therefore, suggests an involvement in GH regulation, especially as GHRH is also synthesized and released from neurons in this nuclei. A physiological role of hypothalamic ghrelin in GH regulation (supported by the presence of ghrelin in

pituitary portal circulation and an inhibition of GH secretion following ghrelin blockade) has, however, yet to be established.

The presence of ghrelin in the avian hypothalamus may also indicate it is involved in the hypophysiotropic regulation of GH secretion, although it is largely present in magnocellular neurons rather than parvocellular neurons, and it is not present in the infundibular nucleus (the avian homologue of the arcuate nucleus). These magnocellular neurons may, however, still communicate with the anterior pituitary gland via the median eminence or via the posterior lobe. The localization of ghrelin in the avian hypothalamus is, however, different from that in mammals and this, may indicate it has different physiological roles.

II. EVOLUTIONARY PERSPECTIVES

It has been postulated that the growth hormone secretagogue receptor (GHS-R) (ghrelin-R) and its natural ligand are highly conserved. The presence of a functional GHS-R has been demonstrated in Pufferfish (*Spherooides nephelus*), since synthetic GHSs (GHRP-6, L-692, 429 and MK-677) activate the pufferfish GHS-R, as measured by ligand-induced intracellular calcium changes involved in GH release (Palyha et al., 2000). These GHSs also activate the human GHS-R in a similar manner. Palyha et al., (2000), therefore, suggested that the ligand activation domain of the GHS-R has been evolutionary conserved from pufferfish to humans (400 million years- Precambrian times), implying that the natural ligand for the GHS-R has also been conserved. This is further supported by the ability of KP-102 (a synthetic hexapeptide) to stimulate GH release in a teleost, the cichlid fish (*Oreochromis mossambicus*) (Shepherd et al., 2000). The results of the study, therefore, suggest that the GHS-R may also exist in teleosts, and a ghrelin-like ligand may be present in these lower vertebrates (Shepherd et al., 2000).

Recently, amphibian ghrelin was identified from the stomach of bullfrogs (Kaiya et al., 2001). Bullfrog ghrelin was isolated using a stable cell line expressing rat GHS-R (CHO-GHSR62), in a similar manner to the isolation of rat and human ghrelin. Three forms of ghrelin were detected in the amphibians, one 28 amino acid form and two 27

amino acid forms, which only possess 30% homology with rat and human ghrelin. A unique difference was observed with bullfrog ghrelin, in comparison to mammalian ghrelin, in that threonine is present at position 3 of the peptide instead of serine. However, threonine 3, like serine 3, is octanoylated. This demonstrates the importance of acylation of the position 3 amino acid hydroxyl group, which seems to be general structure of ghrelin across multiple species. Moreover, the N-terminal amino acid residues are highly conserved, since amino acids at positions 1 and 4-7 are identical in all species examined. Overall, it is suggested that the octanoylation of amino acid at position 3 and the N-terminal amino acids are involved in receptor-binding and/or subsequent signal transduction.

Bullfrog ghrelin is only 30% homologous to rat ghrelin and therefore exhibited minimal GH-releasing effects in rats when intravenously injected. However, bullfrog ghrelin potently stimulates GH secretion from dispersed bullfrog pituitary cells (Kaiya et al., 2001). This indicates that the regulatory role of ghrelin in GH secretion is conserved during phylogenetic evolution, although species-specific differences exist in peptide sequence and receptor activation. It is, however, interesting to note that bullfrog ghrelin is predominantly synthesized in the stomach, as observed in rats, and it is also thought to act on the pituitary gland through the systemic circulation. This novel regulatory mechanism of GH control (through a system outside the hypothalamus), is a first in neuroendocrinology, and challenges current dogma on the regulation of pituitary function. Although other peripheral factors are known to participate in pituitary regulation (e.g. insulin-growth factor-1 (IGF-1) in GH regulation, gondal steroids in gondatropin regulation) they serve as feedback regulators. Ghrelin synthesis and gastrointestinal function are not, however, known to be dependent upon GH action.

III. GHRELIN: THE GHRH IN BIRDS ?

The results of these studies indicate that human ghrelin has potent GH-releasing activity in chicks, and may be more potent than human GHRH. It is, therefore, tempting to speculate that ghrelin may be more important than GHRH in the regulation of GH

secretion in birds. This possibility is supported by the poor GH-releasing activity of chicken GHRH peptides (Harvey, 1999 and unpublished observations), and an inability to locate native GHRH peptides in the chicken hypothalamus (Harvey, 1999). Although ghrelin may not be as potent a GH-secretagogue as TRH, in birds (Harvey, 1990), ghrelin may, like TRH, be more important in GH regulation in avian species than it is in mammals, because GHRH appears to be of little physiological significance in birds (Harvey, 1999).

IV. STRUCTURE-FUNCTION RELATIONSHIPS

The structure of ghrelin is unique, and is the first demonstration of a mammalian peptide, which has an octanoyl group covalently linked to the hydroxyl group of serine at position 3 of the peptide. The octanoylation of serine 3 is essential for ghrelin's GH-releasing ability. The acyl acid responsible for the octanoylation of ghrelin must, therefore, be an important regulator of ghrelin, which needs to be established. Non-octanoylated ghrelin is biologically inactive. The GHS-R is also unique, since it belongs to a new family of G-protein coupled receptors (GPCRs), which is not related to any other known GPCR. This could, therefore, be the beginning of discoveries of a whole new family of peptides (and their respective receptors) that are yet to be isolated. The GHS-R has been cloned and characterized in chickens, but the native ligand has yet to be isolated and sequenced. It would, therefore, be of interest to investigate if chicken ghrelin is also octanoylated. Moreover, the possible existence of U-factors or additional ligands for the GHS-R subtypes needs to be investigated in all species.

The structure of ghrelin is also of interest because of the structural homology that exists between ghrelin, motilin and motilin-related peptide (MTLRP). These similarities may suggest the evolution of these peptides from a common ancestral peptide. It is, however, interesting to note that motilin and MTLRP are not octanoylated. The similarity of ghrelin and motilin raises the possibility that these peptides may, in fact, be the same.

V. FUTURE STUDIES

The present study demonstrates human (h) ghrelin to exhibit potent GH-releasing activity in chickens, and suggests a role for chicken ghrelin in GH release. However, h growth hormone-releasing hormone (GHRH) also increases GH levels in chickens but chicken GHRH is ineffective at promoting GH secretion and it is, therefore, possible that chicken ghrelin may not participate in GH regulation in this species. Furthermore, whereas rat and human ghrelin are potent feeding stimulants in mammals (Wren et al., 2000; Shintani et al., 2001), h ghrelin inhibits feeding in chicks (Furuse et al., 2000). The orexigenic/anorexigenic effects of the chicken ghrelin should, therefore, be determined for comparison. The chicken ghrelin gene should, therefore, be cloned and sequenced, and the coded peptide should be synthesized for physiological testing.

To clone chicken ghrelin, conventional molecular biology techniques such as reverse transcription-polymerase chain reaction (RT-PCR), 3'/5' rapid amplification of cDNA ends (RACE)-PCR and cDNA library screening should be used. Briefly, this involves extracting total RNA from chicken brain tissues, followed by RT-PCR with oligonucleotide primers for conserved regions of the rat/human ghrelin gene to obtain amplified cDNA moieties. These moieties are identified by electrophoresis in agarose gels, and isolated by gel extraction, using commercial reagents. The resulting cDNA fragment should then be subcloned into a vector (e.g. PCR II TOPO vector, Invitrogen, California, USA) for expression. The plasmid, containing the desired cDNA, insert should then be purified (e.g. Plasmid midi-kit, QIAGEN Inc, Canada) and sequenced on an automated sequencer. Gene specific primers for 3' and 5' RACE based on rat/human ghrelin gene that codes for the ghrelin peptide could be utilized, to obtain both 3' and 5' ends of the cDNA. A probe for the complete sequence of ghrelin cDNA can then be synthesized and used for screening a chicken brain genomic library to help identify the chicken ghrelin genomic sequence. Alternatively, to obtain the genomic sequence of chicken ghrelin gene, genomic DNA should be extracted and degenerate primers should be used for performing PCR. After gel electrophoresis, the band could be excised to recover the DNA, this DNA could then be subcloned into a vector and sequenced.

Finally, this sequence can be compared with known sequences of the ghrelin gene in mammals to determine the homology between the ghrelin genes.

The present study demonstrated the presence of a ghrelin-like peptide in a discrete population of hypothalamic nuclei and magnocellular cells, but not in the ARC nucleus, of the chicken brain, it is possible that the absence of ghrelin-immunoreactivity in the ARC nucleus of the chicken brain and the chicken gastrointestinal tract could be due to the presence of splice variants of the chicken gene, that code for proteins that were not recognized by the antibody used. Cloning chicken ghrelin is, therefore, required to address such problems. Alternatively, the technique used in the present study may have been unable to detect low concentrations of ghrelin in either the chicken ARC nucleus or in gastrointestinal tract.

A limitation within the current study that needs to be addressed is the use of a polyclonal antibody in the immunocytochemical studies for investigating the distribution of a ghrelin-like peptide in chickens. This polyclonal ghrelin antibody may have crossreacted with closely related proteins such as motilin. This is, however, unlikely since ghrelin-immunoreactivity was not detected in the chicken stomach or ileum where previous studies have shown motilin to be present in chickens (De Clerq et al., 1996; Ando et al., 2000).

Furthermore, the current study has provided no evidence of the precise location of where these specific nuclei project and extend to. To establish links between the sites of ghrelin synthesis and action, neuronal tracing techniques could be used. A widely used system for neuronal tracing is the 'brain-slice-chamber' (BSC) (Collingridge, 1995), which allows pico-amounts of a tracer to be micro-injected into the population of neurons in question. The neuronal tracer can either be an anterograde tracer, which labels other brain nuclei from which the specific nucleus in question receives information from (i.e. tracing efferent connections). Alternatively, retrograde tracers could be used, which label brain regions that the nuclei project to (i.e. tracing afferent connections). DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindo-carbocyanine perchlorate), a member of the carbocyanine dye family, is known to effectively label neuronal membranes, and functions as both an anterograde and retrograde tracer which travels via lateral diffusion

(Honig & Hume, 1989). The projection of ghrelin neurons to the pituitary gland could be similarly determined by neuronal tracing methods.

It has become apparent that ghrelin is widely distributed in mammalian tissues. In addition to its location in the stomach and hypothalamus of rats and humans (Kojima et al., 1999; Date et al., 2000a,b), ghrelin is also present in the mouse kidney (Mori et al., 2000) and was recently found in human immune tissues (T cells, B cells and neutrophils) (Hattori et al., 2001). It would, therefore, be of interest to investigate the presence of ghrelin in other tissues of the chicken, to fully understand the multifunctional roles of this peptide.

The present study demonstrated a stimulatory effect of mammalian ghrelin on GH secretion in chickens. However, further studies are required to fully characterize its role in GH secretion in birds, particularly studies to assess the possibility that it has additive, synergistic or inhibitory effects with GHRH, TRH and SRIF. It would also be useful to know if ghrelin is specific for GH, especially as bullfrog ghrelin not only increases GH release but stimulates the release of prolactin (Kaiya et al., 2001).

At present, the GHS-R has only been found on chicken pituitary membranes (Toogood et al., 1999; Gaylinn et al., 2000). It would, therefore, be of interest to further investigate the possible presence of the GHS-R in other areas, especially since a widespread distribution of the GHS-R is observed in mammals (Howard et al., 1996; Muccioli et al., 1997; Smith, 1998; Smith et al., 1999a). Furthermore, subtypes of the GHS-R exist in mammals (Muccioli et al., 1997; Ong et al., 1998; Smith et al., 1999b), and the possibility of GHS-R subtypes being present in chickens should also be addressed. If subtypes of the GHS-R are found in the birds, it is possible that more than one ligand for these receptors are also present.

Finally, studies are required to determine if ghrelin has stimulatory effects on GH secretion in extrapituitary sites within the early chick embryo. Although this thesis demonstrated the presence of ghrelin and GH in the same embryonic tissues, it did not provide evidence for a role of ghrelin in stimulating GH release from these cells. Similarly, since GH is found in the proventricularis of the chick embryo (Murphy & Harvey, 2001a,b), it would be of interest to determine if ghrelin is present in this stomach

derivative prior to hatch, since it is not present in the gastrointestinal tract of neonatal chicks after hatch (Ahmed & Harvey, 2001).

Overall, although ghrelin has already bought much excitement into the GH field, there is still a lot to learn about the biochemical and physiological characteristics of this novel hormone.

VI. CONCLUSION

Growth hormone (GH) regulation in mammalian species is thought to be reciprocally controlled by growth hormone-releasing hormone (GHRH) and somatostatin (SRIF), whilst GH regulation in avian species is controlled by thyrotropin-releasing hormone (TRH) and SRIF. The discovery of ghrelin, a potent GH-releasing factor (GRF), has added a new dimension to the dual hypothalamic control of GH in birds and mammals. The present study has detected a ghrelin-like peptide in the chicken hypothalamus and in neural tissues of the early chick embryos, and, stimulation with human ghrelin resulted in potent GH release *in vivo* and *in vitro* in chickens. In conclusion, these results strongly suggest ghrelin participates in GH regulation, acting as a third regulatory factor, in addition to TRH and GHRH, in stimulating GH release in birds.

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Chicken growth hormone (cGH) radioimmunoassay (RIA)

The principles of radioimmunoassay (RIA)

Radioimmunoassay (RIA) involves the reaction of fixed amounts of antibody (anti-cGH) and labelled hormone (^{125}I -cGH; radioactive tracer) in the presence of unknown amounts of hormone (unlabelled) to be measured in samples. Labelled and unlabelled hormones compete for the same antibody-binding site; therefore, when the concentration of unlabelled hormone is high, the amount of labelled binding will be low and *vice versa*. Separation of bound from free radioactivity is used to calculate hormone concentration, since the activity of unknown samples can be compared with the activity of known samples from the standard curves.

The iodination procedure

Chicken (c) GH was iodinated by the Chloramine T method (which results in the labeling of tyrosine residues in the GH molecule with radioactive iodine). The reaction was initiated by the addition of 100 μg chloramine T in 20 μl 0.05M sodium phosphate with 5 μg cGH, and was terminated 60 sec later by the addition of 250 μg sodium metabisulphite in 100 μl of the same phosphate buffer.

Purification of labelled cGH (tracer)

The labelled hormone was isolated from unreacted free iodine and very high molecular weight (damaged and aggregate) protein by gel filtration on sephadex G100. Prior to use, the sephadex column (0.8cm x 24cm) was equilibrated with column buffer (0.15M sodium chloride, 0.01M sodium phosphate, pH 7.5). The reaction mixture from

the iodination procedure was then applied to the column and eluted with the same column buffer. 0.5ml fractions were collected into an equal volume (0.5ml) of buffer and 10ul aliquots of each fraction counted for radioactivity. A typical elution pattern consisting of three major peaks was obtained after subjection of the reaction mixture for gel filtration. The first peak represents aggregated and damaged hormone. The second peak was strongly immunoreactive and co-eluted with monomer GH and was used as the tracer. The third peak represented unreactive free iodine.

Hormone determinations

Chicken GH was measured using a double-antibody RIA technique (Harvey & Scanes, 1977). All assays were performed in polystyrene tubes with a sample volume of 200 μ l and a final assay volume of 400 μ l. All samples were assayed in duplicate. Standard curves were also drawn for each assay using 200 μ l of 1 μ g/ml cGH standard, followed by double dilutions of the standards (range 1-500 μ g/ μ l cGH). GH was measured in a sample volume of 200 μ l and a final incubation volume of 400 μ l. Serial dilutions of a cGH standard ranging from 1 – 500 μ g/l was constructed for each assay. Each assay tube contained, tracer (125 I-cGH; 10,000cpm), 1% NRS, RIA buffer, and primary antiserum (rabbit anti-cGH; final dilution 1:25,000; AFP55111186Rb, NIDDK, USA). After an overnight incubation, at 4°C, the secondary antibody (anti-goat IgG, whole molecule, Sigma) was added at a final dilution of 1:50. After an incubation for 48 h at 4°C, the tubes were centrifuged at 2700g at 4°C for 30 min and 5% (w/v) corn starch was added to cover the precipitate, After further centrifugation (2700 g at 4°C for 10 min) the supernatants were discarded and pellet radioactivity was counted using a LKB gamma-master (Wallac OY, Turku, Finland).

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